Defences of *Helicobacter* species against host antimicrobials

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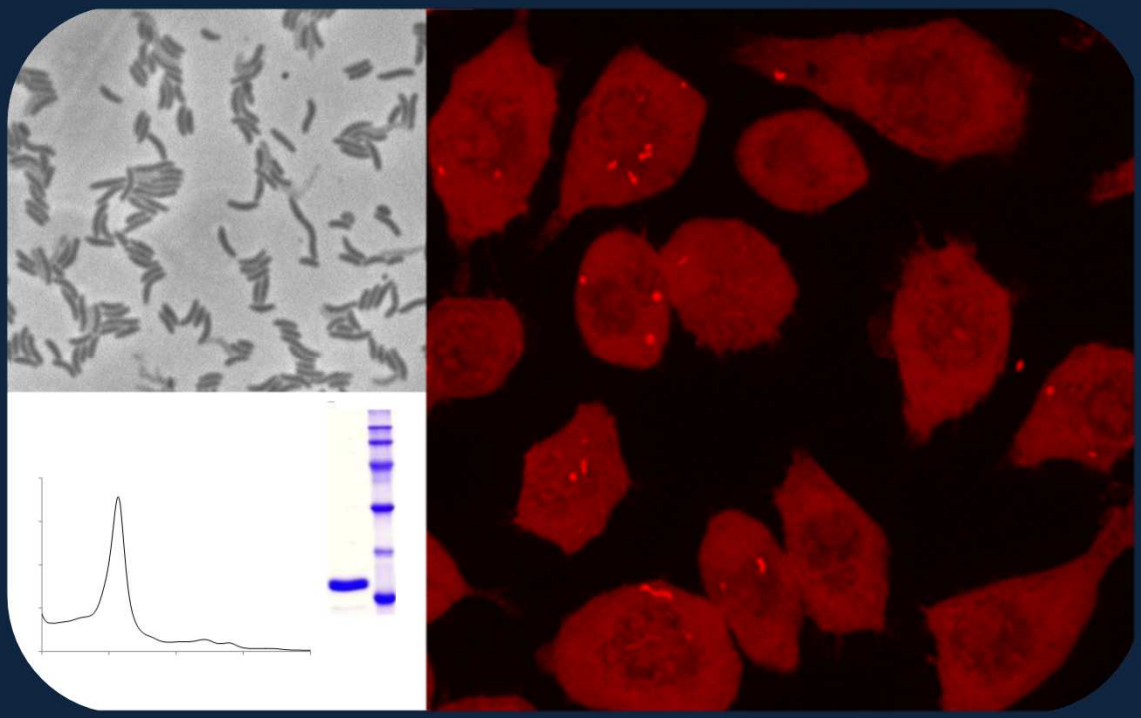

Dissertation presented to obtain the Ph.D degree in Biochemistry

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

Oeiras,
June, 2016

UNIVERSIDADE NOVA DE LISBOA

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From left to right: Mónica Oleastro (4th oponente), Gabriel Martins (3rd opponent), **Marta Justino (Co-supervisor)**, Miguel Viveiros (2nd opponent), **Adelina Margarida Parente**, **Lígia Saraiva (supervisor)**, Cecília Arraiano (president of the jury), and Maria do Céu Figueiredo (1st opponent).

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“Science knows no country, because knowledge belongs to humanity, and is the torch which illuminates the world”

Louis Pasteur

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This thesis is dedicated to my parents and husband

Thesis Publications

This dissertation is based on the following original publications, listed by chronological order:

Justino MC, **Parente MR**, Boneca IG, Saraiva LM. FrxA is an S-nitrosoglutathione reductase enzyme that contributes to Helicobacter pylori pathogenicity. FEBS J. 2014 Oct. 281(19):4495-505.

Parente MR, Monteiro JT, Martins GG, Saraiva LM. Helicobacter pullorum induces in murine macrophages nitric oxide release that promotes phagocytosis and killing. Microbiology. 2016 Jan 13. doi: 10.1099/mic.0.000240.

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Margarida R. Parente, Elena Forte, Micol Falabella, João T. Monteiro, Ivo G. Boneca, Miguel Teixeira, Alessandro Giuffrè and Lígia M. Saraiva (2016) The nitrosative stress defences of the enterohepatic Helicobacter pullorum.

Publications not included in this thesis:

Tavares AF, **Parente MR**, Justino MC, Oleastro M, Nobre LS, Saraiva LM. The bactericidal activity of carbon monoxide-releasing molecules against Helicobacter pylori. PLoS One. 2013 Dec. 26;8(12):e83157.

Abstract

Bacteria of the genus *Helicobacter* are related to gastrointestinal and hepatic disease in humans. *Helicobacter pylori* infects the gastric mucosa of a large percentage of the human population and has been reported to cause gastric ulcer and stomach cancer. Other *Helicobacter* related species, such as the enterohepatic *Helicobacter pullorum*, although firstly discovered in chickens are now known to be associated with human digestive disorders such as gastroenteritis, inflammatory bowel disease, hepatobiliary disease and hepatic cancer.

During host invasion, all pathogens are exposed to the antimicrobials produced by the innate immunity's phagocytes such as nitric oxide (NO) and derivate reactive nitrogen species (RNS), which constitute one of the main strategies employed by the host to eliminate pathogens. Due to the high importance of *H. pylori* as a human pathogen and the more recent recognition of *H. pullorum* as an emergent human pathogen, the so far unknown mechanisms used by these bacteria to circumvent the innate immune system need to be addressed. Therefore, the objectives of this work were: i) investigation of the role of *H. pylori* nitroreductases in the defence against the host induced nitrosative stress, ii) evaluation of the sensitivity of *H. pullorum* to antimicrobials, iii) assessment of the ability of *H. pullorum* to activate, infect and survive within macrophages, and iv) identification and characterization of the enzymes putatively involved in *H. pullorum* defence against host antimicrobials.

H. pylori nitroreductases activate metronidazole, which is the antibiotic commonly used for *H. pylori* treatment. In this work, a new function of these enzymes was explored. For this purpose, an *H. pylori* strain deleted in the nitroreductase *frxA* gene was constructed, its phenotype and the expression of the *frxA* gene was analysed under NO stress conditions. Inactivation of the *frxA* gene resulted in a strain that is more sensitive to nitrosative stress. Furthermore, *H. pylori frxA* is induced in response to nitrosative stress generators. Studies on the capacity of the wild type and *frxA* deficient cell extracts to degrade the toxic compound nitrosoglutathione (GSNO)

showed that in the absence of *frxA H. pylori* cells have significantly lower GSNO reductase activity. Accordingly, experiments on the activity of the purified FrxA protein towards nitrocompounds and S-nitrosothiols revealed that not only FrxA reduces metronidazole, but exhibits a GSNO reductase activity. The interaction of *H. pylori frxA* mutant with macrophages and mice was also analysed. FrxA was shown to contribute to the survival of *H. pylori* within macrophages and to the bacterium´s virulence during mice colonization.

The resistance of the enterohepatic *H. pullorum* to nitrosative stress was also analysed. *H. pullorum* was exposed at different growth phases to nitrosative stress generated by several NO donors. Treatment with NO lowered the *H. pullorum* viability, in a growth-phase-dependent manner and, *H. pullorum* cells suffered a decrease in the mean bacterial cell size. In the second part of this study, the interaction between *H. pullorum* and host cells was analysed. Confocal microscopy revealed that *H. pullorum* is internalized by murine macrophages, triggering the production of NO that promoted phagocytosis and bacterial clearance. Interaction between *H. pullorum* and macrophages was shown to stimulate secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and MIP-2, indicating that *H. pullorum* triggers a host inflammatory response.

To identify the nitrosative protective genes of *H. pullorum* a sequence comparison analysis of its genome was performed. Five gene products were selected and studied. To this end, their expression pattern was analysed, mutant strains were constructed and characterized under stressed conditions. Furthermore, the survival of the mutants was studied in phagocytic cells such as macrophages. Finally, all the respective proteins were recombinantly produced, purified and biochemically characterized. We show that *H. pullorum* contains two haemoglobins, namely, a single domain and a truncated haemoglobin, which genes are induced by GSNO. Complementation studies showed that the *H. pullorum* haemoglobins abolished the nitrosative stress sensitive phenotype of an *E. coli* flavohaemoglobin mutant. Furthermore, *H. pullorum* single domain globin increased the resistance to NO stress and improved the ability of *H. pullorum* to survive within macrophages. On the contrary, the deletion of the truncated globin did not alter the sensitivity of *H. pullorum* to nitrosative stress and macrophage killing.

The analysis of *H. pullorum* genome also revealed the presence of homologs of peroxiredoxins, named Prx1, Prx2 and Prx3. Expression of *prx1* was not modified by oxidative and nitrosative stresses. *Prx2* and *prx3* were induced in response to peroxynitrite and hydrogen peroxide, respectively. Whereas the *prx1* mutant did not show susceptibility to oxidative and nitrosative stresses the *prx2* mutant showed a significant growth defect even under normal growth conditions. *H. pullorum prx1* mutant showed a survival to macrophage killing similar to the wild type strain. Furthermore, purified *H. pullorum* peroxiredoxins were shown to detoxify peroxynitrite, having Prx3 the highest activity. Therefore it was concluded that peroxiredoxins contribute to nitrosative stress protection of *H. pullorum*. Data also suggest that *prx3* may be an essential gene of *H. pullorum*.

In summary, *H. pylori* nitroreductase FrxA was shown to protect from nitrosative stress injury due to its ability to reduce S-nitrosothiols, and to contribute for the pathogen´s virulence. *H. pullorum* was shown for the first time to activate and to be internalised by macrophages, and to have a decreased viability when exposed to NO producing macrophages. Furthermore, proteins that confer resistance to *H. pullorum* when facing RNS, such as a single domain haemoglobin, were unveiled.

Altogether this study contributed to further understand the defence mechanisms used by *Helicobacte*r species against the antimicrobials produced by the host immunity. This study also provided new insights about the ability of *H. pullorum* to trigger host inflammation and to survive upon contact with macrophages.

Resumo

As bactérias do género *Helicobacter* estão relacionadas com doenças gastrintestinais e hepáticas em seres humanos. *Helicobacter pylori* infecta a mucosa gástrica de uma grande percentagem da população humana, e é o agente responsável pelo desenvolvimento de úlcera gástrica e cancro do estômago. Outras bactérias do género *Helicobacter*, tal como a espécie enterohepática *Helicobacter pullorum*, embora de origem aviária, tem sido associada a doenças digestivas humanas como gastroenterite, doença inflamatória do intestino, doença hepatobiliar e cancro hepático.

Durante a invasão do hospedeiro, os organismos patogénicos são expostos a agentes antimicrobianos produzidos pelos fagócitos do sistema imune inato, nomeadamente o óxido nítrico (NO) e as espécies reativas de nitrogénio (ERN) derivadas, que constituem uma das principais estratégias do hospedeiro para eliminar os organismos patogénicos. Devido à elevada importância de *H. pylori* como um patogénico humano e ao recente reconhecimento de *H. pullorum* como um agente patogénico humano emergente, os mecanismos utilizados por estas bactérias para contornar o sistema imune inato, que embora até agora desconhecidos, necessitam de ser compreendidas. Assim, os objetivos deste trabalho incluíram: i) investigação do papel das nitroredutases de *H. pylori* na defesa contra o stress nitrosativo gerado pelo hospedeiro, ii) avaliação da sensibilidade de *H. pullorum* aos agentes antimicrobianos, iii) apreciação da capacidade de *H. pullorum* para ativar, infetar e sobreviver dentro dos macrófagos, e iv) identificação e caracterização de enzimas com potencial para estar envolvidas na defesa de *H. pullorum* contra os agentes antimicrobianos produzidos pelo hospedeiro.

As nitroredutases de *H. pylori* ativam o metronidazol, que é o antibiótico normalmente usado para o tratamento de *H. pylori*. Neste estudo, uma nova função para estas enzimas foi explorada. Para este efeito, foi construída uma estirpe de *H. pylori* deletada no gene da nitroredutase *frxA*, o seu fenótipo e a expressão do gene

frxA foi analisada sob condições de stress. A inativação do gene *frxA* resultou numa estirpe que é mais sensível ao stress nitrosativo. Verifica-se também que o gene *frxA* de *H. pylori* é induzido em resposta aos agentes geradores de stress nitrosativo. Estudos sobre a capacidade de degradação do composto tóxico nitrosoglutationa (GSNO) na estirpe selvagem e na estirpe deficiente em *frxA* mostraram que na ausência de *frxA* as células têm significativamente menor capacidade para reduzir GSNO. Assim, as atividades enzimáticas da proteína FrxA para nitrocompostos e para S-nitrosotióis foram realizadas, e revelaram que FrxA não só reduz metronidazol mas exibe também uma atividade de GSNO redutase. A interação da estirpe de *H. pylori* mutada no gene *frxA* com macrófagos e ratinhos foi também analisada. FrxA mostrou contribuir para a sobrevivência de *H. pylori* no interior de macrófagos e para a virulência da bactéria durante a colonização de ratinhos.

A resistência da bactéria enterohepática *H. pullorum* ao stress nitrosativo foi também analisada. Em diferentes fases do seu crescimento, *H. pullorum* foi exposta ao stress nitrosativo gerado por vários dadores de NO. O tratamento com NO reduziu a viabilidade de *H. pullorum*, de um modo dependente da fase de crescimento bacteriana, e promoveu uma diminuição no tamanho médio das células de *H. pullorum*. Na segunda parte deste estudo, foi analisada a interação entre as células de *H. pullorum* e do hospedeiro. Experiências de microscopia confocal mostraram que *H. pullorum* é internalizada por macrófagos de ratinho e que induz a produção de NO, que por sua vez promove a fagocitose e a eliminação da bactéria. A interação entre *H. pullorum* e os macrófagos resultou na indução da secreção de citoquinas pró-inflamatórias, tais como TNF-α, IL-1β, IL-6 e de MIP-2, indicando que a *H. pullorum* desencadeia uma resposta inflamatória no hospedeiro.

A análise da comparação de sequências do genoma de *H. pullorum* permitiu identificar cinco genes envolvidos na proteção de *H. pullorum* contra o stress nitrosativo. O padrão de expressão destes genes foi analisado e as estirpes mutantes nestes genes foram construídas e caracterizadas em condições de stress. Além disso, a sobrevivência dos mutantes foi estudada em células fagocíticas, tais como macrófagos. Por último, as respetivas proteínas recombinantes foram purificadas e caracterizadas bioquimicamente. Mostrou-se que *H. pullorum* contém duas hemoglobinas, nomeadamente uma hemoglobina “single-domain” e uma

hemoglobina truncada, cujos genes são induzidos por GSNO. Estudos de complementação mostraram que a expressão das hemoglobinas de *H. pullorum* suprime o fenótipo de sensibilidaade ao stress nitrosativo de uma estirpe de *E. coli* deletada no gene flavohemoglobina. Além disso, a hemoglobina "single-domain" de *H. pullorum* aumenta a resistência ao stress nitrosativo e melhora a capacidade de *H. pullorum* para sobreviver em macrófagos. Pelo contrário, a ausência da hemoglobina truncada não alterou a sensibilidade de *H. pullorum* ao stress nitrosativo e aos macrófagos.

A análise do genoma de *H. pullorum* revelou também a presença de potenciais homólogos de peroxiredoxinas, que foram denominados Prx1, Prx2 e Prx3. O estudo da expressão do gene *prx1* mostrou que este não é alterada pelos stresses oxidativo e nitrosativo. Os genes *prx2* e *prx3* são induzidos em resposta ao peroxinitrito e ao peróxido de hidrogénio, respetivamente. Enquanto que o mutante *prx1* não apresentou suscetibilidade aos stresses oxidativo e nitrosativo, o mutante *prx2* apresenta um crescimento deficiente mesmo sob condições de não stress. A estirpe de *H. pullorum* mutada no gene *prx1* tem níveis de sobrevivência no interior de macrófagos semelhantes aos da estirpe selvagem. As peroxiredoxinas de *H. pullorum* purificadas mostraram ser destoxificantes de peroxinitrito, tendo Prx3 a maior atividade enzimática. Por conseguinte, conclui-se que as peroxiredoxinas contribuem para a proteção de *H. pullorum* contra o stress nitrosativo. Os dados também sugerem que *prx3* deverá ser um gene essencial para *H. pullorum*.

Em resumo, foi mostrado que a nitroredutase FrxA de *H. pylori* protege a bactéria de lesões geradas pelo stress nitrosativo, devido à sua capacidade de reduzir a S-nitrosotióis, e contribuiu também para a virulência do organismo patogénico. Também pela primeira vez, foi mostrado que *H. pullorum* é internalizada por macrófagos e promove a sua ativação. *H. pullorum* mostrou ter uma viabilidade diminuída quando exposta ao NO produzido pelos macrófagos. As proteínas que conferem resistência a *H. pullorum* contra o stress nitrosativo, tal como a hemoglobina "single-domain", foram estudadas.

Este estudo contribuiu para um melhor conhecimento dos mecanismos de defesa utilizados pelas espécies *Helicobacter* contra os agentes antimicrobianos produzidos

pelo sistema imunitário do hospedeiro. Este trabalho forneceu também novos dados acerca da capacidade de *H. pullorum* para promover uma inflamação no hospedeiro e sobreviver quando entra em contato com os macrófagos.

Abbreviations list

Δ	Deletion
AhpC	Alkyl hydroperoxide reductase C
Arg2	Arginase II
BA	Blood agar
BB	Brucella Broth
BCA	Bicinchoninic acid method
Bcp	Bacterioferitin comigratory protein
BHI	Brain heart infusion
βCD	β-cyclodextrin
CagA	Cytotoxin associated gene A
CDT	Cytolethal distending toxin
CFU	Colony forming units
C_P	Peroxidatic cysteine
C_R	Resolving cysteine
CuZn-Sod	Copper-zinc Sod
FAD	Flavin adenine dinucleotide
FCS	Fetal calf serum
Fe-S	Iron-sulphur
Fe-Sod	Iron Sod
FMN	Flavin mononucleotide
DNA	Deoxyribonucleic acid
Dps	DNA binding proteins from starved cells
DNICs	Dinitrosyl iron complexes
DTT	Dithiothreitol
DTPA	Diethylenetriamine pentaacetic acid
e-	Electron
EHS	Enterohepatic *Helicobacter* species
EPR	Electron paramagnetic resonance
G + C	Guanine plus cytosine
GHS	Gastric *Helicobacter* species
GSNO	S-nitrosoglutathione
GSNOR	GSNO Reductase
gyrA	Gyrase A
hmp	Flavohaemoglobin
IBD	Intestinal bowel disease
i.e.	*id est*, that is to say
INF	Interferon
IL	Interleukin
iNOS	Inducible nitic oxide synthase

IPTG	Isopropyl-β-D-thiogalactopyranoside
LMW	Low molecular weight
L-NMMA	NG-Monomethyl-L-arginine acetate salt
LB	Luria-Bertani
LPS	Lipopolysaccharides
MALT	Mucosa-associated lymphoid tissue
MIP-2	Macrophage inflammatory protein 2
Mn-Sod	Manganese Sod
MOI	Multiplicity of infection
MPO	Myeloperoxidase
Msr	Methionine sulphoxide reductase
NADH	β-Nicotinamide adenine dinucleotide, reduced form
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced form
NapA	Neutrophil activating protein A
Ni-Sod	Nickel Sod
NO	Nitric oxide
NOR	NO reductases
NOX	Phagocyte NADPH oxidase
NF-kβ	Nuclear factor-kβ
NOS	Nitric oxide synthases
OD_{600}	Optical density at 600 nanometers
ODC	Ornithine decarboxylase
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffer
PCR	Polymerase chain reaction
PPI	Proton pump inhibitor
Prx	Peroxiredoxin
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polimerase chain reaction
SDS	Sodium dodecyl sulfate
SMOX	Spermine oxidase
Sod	Superoxide dismutase
Sor	Superoxide reductase
spp.	Species
Th	T helper
TLRs	Toll-like receptors
Tpx	Thiol peroxidase
Treg	Regulatory T cells
Trx	Thioredoxin
TrxR	Thioredoxin reductase
VacA	Vacuolating cytotoxin A
YID	Iodotyrosine deiodinase

Strains

B. subtilis	*Bacillus subtilis*
B. abortus	*Brucella abortus*
C. jejuni	*Campylobacter jejuni*
D. vulgaris	*Desulfovibrio vulgaris Hildenborough*
E. faecalis	*Enterococcus faecalis*
E. coli	*Escherichia*
F. tularensis	*Francisella tularensis*
H. influenzae	*Haemophilus influenzae*
H.	*Helicobacter*
L. monocytogenes	*Listeria monocytogenes*
M.	*Mycobacterium*
N. gonorrhoeae	*Neisseria gonorrhoeae*
P. gingivalis	*Porphyromonas gingivalis*
P. aeruginosa	*Pseudomonas aeruginosa*
P. haloplanktis	*Pseudoalteromonas haloplanktis*
R. eutropha	*Ralstonia eutropha*
R. capsulatus	*Rhodobacter capsulatus*
S. cerevisiae	*Saccharomyces cerevisiae*
S. typhimurium	*Salmonella enterica serovar typhimurium*
S. aureus	*Staphylococcus aureus*
S. pneumonia	*Streptococcus pneumonia*
V.	*Vibrio*

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Introduction

Chapter I

Oxidative and Nitrosative Stress as Innate Immunity Weapons to Fight Pathogens

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1.1 Innate and Adaptive Immunity

The mammalian immune system is divided into two distinct yet interrelated subsystems: the innate and the adaptive immunity. The innate immunity operates at the early stages of infection through nonspecific recognition of a pathogen and subsequent activation of the adaptive immune response. The cells involved in innate immunity are: macrophages, dendritic cells, mast cells, neutrophils, eosinophils, natural killer, and epithelial cells. Contrary to the innate immunity, the adaptive immunity involves specific recognition of a pathogen, due to a vast repertoire of antigen-specific recognition receptors of its effector cells, the T and B lymphocytes. A specific property of the adaptive immunity is its memory, which is generated after a first contact of B and T cells with the pathogen that leads to their differentiation in memory B cells and T cells. These memory cells are long lived and in subsequent encounters with the same pathogen originate a faster and enhanced immune response. Depending on the type of cells employed, the adaptive immunity can also be divided in type 1 immunity, which is usually involved in the elimination of bacteria, viruses and other microbes and type 2 immunity, mainly involved in multicellular parasites and allergens responses (1, 2).

The innate immune system uses several pattern recognition receptors, like Toll-like receptors (TLRs), C-type lectin receptors and NOD-like receptors to detect common structural and functional features associated with different classes of microorganisms, the so-called pathogen associated molecular patterns (PAMPs). In a general view, the immune system presents the following common principle to respond to an invader organism: PAMPs recognition by the innate immunity results in a primary innate response which includes the production of a first set of cytokines (level 1 cytokines). The profiles of level 1 cytokines produced depend on several factors, such as the compartment in which the bacterial PAMP is recognized, microorganism viability and virulence, which leads to different types and intensities of the adaptive and effector responses. For example, bacteria detected in the lumen of the intestine usually do not trigger inflammation, whereas those that have crossed the epithelium induce a local inflammation, and bacteria in the bloodstream, which represents a higher level of danger for the host, originate a strong systemic response.

Then, level 1 cytokines trigger specific B lymphocytes recruitment and development of specific T helper (Th) phenotypes. Once activated, adaptive cells, produce a second set of cytokines (level 2 cytokines) that potentiate the innate effector responses (1). In the case of type 1 immune responses, which are related to bacterial elimination, level 1 cytokines produced include interleukin (IL)-12, IL-23, IL-6 and IL-1β and level 2 cytokines include interferon (INF)-γ, IL-17 and IL-22 (1).

Phagocytosis is one of the most important effector mechanisms of the innate immunity to eliminate pathogens. Phagocytosis consists in the recognition, ingestion, digestion, and elimination of microorganisms, "non-self" particles and damaged or apoptotic cells from the host. Receptor-mediated internalization occurs through an actin-dependent zippering of the phagocyte membrane around the antigen, forming a cup that leads to progressive microorganism engulfment in a phagocytic vacuole or phagosome. This compartment then interacts and fuses sequentially with early endosomes, late endosomes and finally with lysosomes, culminating in the formation of the phagolysosome. These vesicles provide a closed hostile environment to microorganisms, as they are exposed to low pH, low iron and nutrient availability, and to an array of bactericidal molecules such as antimicrobial peptides, reactive oxygen species (ROS), produced by the phagocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX), and reactive nitrogen species (RNS) generated by the inducible nitic oxide synthase (iNOS). When bacterial enzymatic defences are not enough to completely scavenge the ROS and RNS, the so-called oxidative and nitrosative stresses, these chemical species react with bacterial biomolecules causing their damage (see below). In addition to their role in microorganisms' engulfment and killing, phagocytes are also responsible to present the digested microorganisms to lymphocytes to activate the adaptive response (3-6). In particular, dendritic cells play an important role in directing the nature of the adaptive immune response, since these cells are the main responsible for the priming of naive T cells in effector T cells, such as Th1, Th17, and Th2 through antigen presentation and also by co-stimulatory signals and T cell inducing cytokines production. On the contrary, when dendritic cells receive tolerance-promoting signals these cells play important roles in establish and maintain the immunological tolerance by inducing the priming of regulatory T cells (Treg) instead of T effector cells (1, 7).

The ROS and RNS production has been shown to be essential for efficient bacterial killing. For example, the deletion in mice of either iNOS or NOX resulted in animals more susceptible to infection by different pathogens (8-12), and, the deletion of the two enzymes leads to a substantial mice death caused by spontaneous infections arising from endogenous flora (13). Furthermore, defects in human genes that encode different subunits of NOX result in chronic granulomatous disease, which is characterized by the occurrence of recurrent infections and a reduced life expectancy (14).

1.2 Oxidative and nitrosative stresses generated in phagocytes

The generation of ROS in phagocytes is initiated by NOX, through the one electron reduction of oxygen to the high reactive radical superoxide in the presence of NADPH. Activation of NOX occurs in response to binding of microorganisms or soluble inflammatory mediators to specific receptors on the phagocyte and involves translocation and assembly of several NOX cytosolic components (p40phox, p47phox, p67phox, and the Rho GTPase, Rac) to the NOX membrane-bound flavocytochrome b_{558} complex (p91phox-p22phox) present in the granules fused to phagosomes (15, 16). The generation of superoxide leads to the production of others ROS. Namely, a further one electron reduction of superoxide catalysed by the scavenger enzyme superoxide dismutase (Sod) (17, 18) or the spontaneous dismutation of superoxide with another superoxide molecule, originates hydrogen peroxide (Reaction 1.1).

Hydrogen peroxide is next converted into the highly reactive hydroxyl radical and hydroxide ion by the iron-catalysed Fenton reaction (Reaction 1.2) or used by myeloperoxidase (MPO) together with chloride in neutrophils to synthesize the highly toxic hypochlorous acid (Reaction 1.3) (Figure 1.1) (19). In addition, the hydroxyl radical is generated by the reaction of superoxide with hypochlorous acid (Reaction 1.4).

Reaction 1.1 $2O_2^{-} + 2H^{+} \rightarrow H_2O_2 + O_2$

Reaction 1.2 $H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^{-} + Fe^{3+}$

Reaction 1.3 $Cl^{-} + H_2O_2 \rightarrow OCl^{-} + H_2O$

Reaction 1.4 $HOCl + O_2^{-} \rightarrow O_2 + Cl^{-} + OH^{\bullet}$

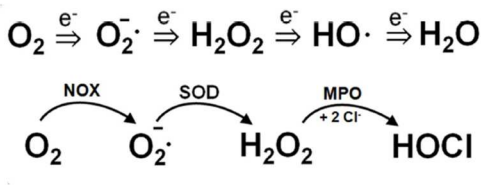

Figure 1.1 Generation of ROS and hypochlorous acid in biological systems. Adapted from (20).

Nitrosative stress is generated by nitric oxide (NO) and derived species. NO is a diatomic gas that is soluble in aqueous systems and freely diffusible across biological membranes. Furthermore, NO is a radical that stabilizes its unpaired electron by reacting with species containing other unpaired electrons or by interacting with the d-orbitals of transition metals (21, 22). In animal tissues, NO is generated enzymatically by nitric oxide synthases (NOS), which in the presence of NADPH, convert L-arginine and oxygen into L-citrulline and NO (23). There are three types of NOS: two constitutively expressed, the endothelial and neuronal NOS, and an inducible enzyme, iNOS. The latter, is expressed in phagocytes and is mainly regulated at the transcriptional level, particularly, in response to microorganisms and pro-inflammatory molecules (24).

NO is not particularly susceptible to oxidation or reduction. Instead, NO reacts: i) with oxygen species such as superoxide and molecular oxygen to form several types of RNS; ii) directly with the formed RNS; and iii) directly with metal centres. In particular, the reaction of NO with oxygen results in the generation of the strong oxidant radical nitrogen dioxide, which by reacting with another NO molecule gives dinitrogen trioxide that in aqueous systems reacts with water to give two equivalents of nitrite (Reaction 1.5 – 1.7). NO also reacts with superoxide yielding

the potent oxidant peroxynitrite (Reaction 1.8), which may decompose to nitrogen dioxide and hydroxyl radical, and that in the presence of carbon dioxide is converted to nitrate and carbonate (25).

Reaction 1.5 $2NO + O_2 \rightarrow 2NO_2$

Reaction 1.6 $NO + NO_2 \rightarrow N_2O_3$

Reaction 1.7 $N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+$

Reaction 1.8 $NO + O_2^- \rightarrow ONOO^-$

1.3 Biological targets of oxidative and nitrosative stress

The effect of ROS and NO in biological systems is related to its concentrations. Moderate quantities of ROS have important regulatory functions in several cellular processes, such as cardiovascular function (26), cell cycle (27) and cell survival (28). In similarity, at concentrations as low as the ones produced by the constitutive neuronal and endothelial NOS, NO has been shown to be involved in biological processes such as signalling, vasodilatation, inhibition of platelet aggregation, blood pressure regulation and neurotransmission. On the contrary, when produced at high concentrations, NO, derived RNS and ROS, have bacteriostatic and bactericidal properties, promoting DNA damage, lipid peroxidation and inactivation of key metabolic enzyme (21-23).

The most common forms of damages in DNA by oxidative stress are strand breaks caused by the attack of hydroxyl radicals to nucleotide sugar moieties and formation of 8-oxoguanine generated by guanine oxidation (29). It has been shown that some RNS, such as nitrogen dioxide, nitrogen trioxide and peroxynitrite, can also directly damage DNA through one-electron oxidation, causing several DNA lesions such as DNA bases deamination and strand breaks (30, 31).

Relatively to lipids, among the ROS, superoxide and hydroxyl radical are able to initiate the process of lipid peroxidation in membranes, damaging polyunsaturated fatty acids, which increases the membrane fluidity and causes loss of function (29). Similarity, peroxynitrite and nitrogen dioxide are strong oxidants capable of initiating lipid peroxidation (32, 33). Amino acid residues such as

cysteine, methionine, tyrosine, phenylalanine and tryptophan react with ROS, NO and RNS leading to functional modifications of proteins (20, 25, 34). Due to the high reactivity of their thiol groups, cysteines are the protein residues most affected. Cysteine thiols are firstly reversibly oxidized to protein disulfides and then irreversibly overoxidized to sulfinic ($R\text{-}SO_2H$) or sulfonic ($R\text{-}SO_3H$) acids by ROS (20). The reaction between cysteine and RNS or NO leads to the formation of reversible S-nitrosothiols by two mechanisms. In the first mechanism, thiolates in redox active cysteines are strong nucleophiles that react with the nitrosonium cation to form S-nitrosothiols. Transnitrosation reactions from low-molecular weight S-nitrosothiols, dinitrosyl iron complexes (DNICs) (see below), and dinitrogen trioxide are the donors of nitrosonium. In the second, S-nitrosothiols are generated through the reaction of thiyl and NO radicals. Nitrogen dioxide is proposed to mediate the abstraction of an electron from the thiol to form the thiyl radical (Figure 1.2) (35). Some bacterial cells use low molecular peptides, as thiol-redox buffer, to maintain the reduced state and alleviate the nitrosative stress within a cell. Glutathione, for example, is a low molecular weight tripeptide composed by glutamate, cysteine and glycine that can be converted to S-nitrosoglutathione (GSNO), and then back converted to glutathione by endogenous GSNO reductases (also known as alcohol dehydrogenase 3, class III alcohol dehydrogenase and glutathione-dependent formaldehyde dehydrogenase) or by the thioredoxin system (36), with the release of less toxic products. However, the generation of large amounts of NO in mammalian cells leads to the formation of large quantities of GSNO molecules, which are nitrosative stress generators by performing nitrosonium groups transfer to bacterial protein thiols (20, 35). Another posttranslational protein modification formed in the presence of NO is nitrotyrosine ($Tyr\text{-}NO_2$). Peroxynitrite, for example, have shown to react with tyrosine residues of some enzymes such as glutamine synthase (37, 38) and ribonucleotide reductase (39), leading to the formation of nitrotyrosine and to their inhibition of function.

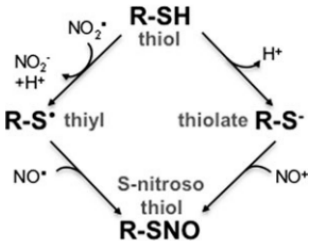

Figure 1.2 Biochemistry of S-nitrosothiols formation in biological systems (35).

Iron-sulphur (Fe-S) clusters are targets of ROS and RNS. Hydrogen peroxide oxidizes solvent exposed 4Fe-4S clusters of redox enzymes resulting in the loss of one or more iron ions, and consequent inactivation of the enzymes. Moreover, the release of free iron contributes to increase the oxidative stress via the Fenton reaction (19). NO reaction with protein Fe-S clusters generates DNICs, which are shown to inhibit the activity of Fe-S cluster containing enzymes such as aconitase, nicotinamide adenine dinucleotide (NADH) and succinate dehydrogenases (40, 41). Moreover, as stated above, DNICs and haem-bond NO groups also mediate the formation of S-nitrosothiols through transnitrosation of nitrosonium equivalents from nitrosylated metals to protein thiols (42). Additionally, NO reacts readily with haem iron centers of proteins like cytochrome *bd* or *bo'* complexes, the two terminal oxidases in *Escherichia coli*, inhibiting oxygen respiration (43). In the same way, NO binds to the haem center of catalase inhibiting its function (41, 44).

In response to the nitrosative and oxidative stresses, bacterial pathogens have evolved mechanisms to sense oxygen and NO and respond through defensive mechanisms that detoxify ROS and RNS and repair damaged cell components (45) (see chapter III). Bacterial nitroreductases, globins and peroxiredoxins (see chapter III, section 3.3-3.5) that have a protective role on *Helicobacter* were studied in this thesis and are presented in chapters IV and VI.

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Chapter II

Helicobacter species

2.1 General characteristics and classification

The *Helicobacter* genus belongs to the class *Epsilonproteobacteria*, order *Campylobacterales*, and family *Helicobacteraceae*. The genus is composed of Gram-negative, microaerobic, non-spore-forming bacteria with curved, spiral or fusiform morphology, typically with 0.2 to 1.2 μm diameter and 1.5 to 10 μm long. (1). A coccoid form can also occur in some *Helicobacter* spp. upon accumulation of toxic compounds in the culture media, by extended incubations periods and other ambient stress factors (2-4). The presence of single or multiple flagella confers mobility to *Helicobacter* cells (1).

Since the discovery of *Helicobacter pylori* in 1983 (5) several other naturally occurring species have been isolated and characterized. The *Helicobacter* genus comprises currently 36 species (http://www.bacterio.net/helicobacter.html), with several other proposed new organisms in the process of being characterized. *Helicobacter* spp. are separated into two categories based on the colonization niche: the gastric *Helicobacter* species (GHS), which colonize the gastric mucosa and proximal duodenum of mammals and the enterohepatic *Helicobacter* species (EHS) that colonize the intestine and/or the hepatobiliary system of mammals and birds (6, 7). However, the same organism can be frequently or occasionally found in both niches, as is the case of *H. aurati* and *H. bilis*, respectively (8). In addition, due to the high level of phenotypic and genotypic similarity of several GHS including *H. suis*, *H. felis*, *H. bizzozeronii*, *H. salomonis*, *H. cynogastricus*, *H. baculiformis* and *H. heilmannii*, these organisms were grouped as *H. heilmannii* sensu lato. The name *H. heilmannii* sensu stricto was attributed to the species isolated in 2012 by Smet and co-workers (9) that had been previously nominated as *H. heilmannii* (10).

Helicobacter species have a relatively small genome of 1.5-2.5 Mb with a guanine plus cytosine (G + C) percentage ranging from 30 to 48%. Until now, the complete genome sequence of a small number of *Helicobacter* species was described (1, 8). The current known *Helicobacter* species, respective hosts and genome sequence status are listed in Table 2.1.

Table 2.1 Classification of *Helicobacter* species, respective hosts and genome projects.

Gastric		
Helicobacter taxon	**Host**	**Genome Project**
H. acinonychis	Cheetah, lion, tiger (11)	PRJNA58685, PRJNA17251 [C]
H. baculiformis	Cat (13)	NA
H. bizzozeronii	Human (15), cat (16), fox, lynx (17), dog (18), cheetah (19)	PRJNA68141, PRJEA65019 [C]
H. cetorum	Dolphin, whale (24)	PRJNA162217, PRJNA97501 [C]
H. cynogastricus	Dog (25)	NA
H. felis	Cat, dog (28), human (29)	PRJNA61409, PRJEA61189 [C]
H. heilmannii sensu stricto	Cat (9), human, fox, lynx (17), dog, non-human primates (33)	PRJNA182935 PRJEB367
H. himalayensis	Marmot (34)	NA
H. mustelae	Ferret, mink (38)	PRJNA46647, PRJEA40677 [C]
H. nemestrinae	Pig tailed macaque (40)	NA

Enterohepatic		
Helicobacter taxon	**Host**	**Genome Project**
H. anseris	Wild goose (12)	NA
H. aurati	Hamster (14)	NA
H. bilis	Mouse (20), dog, cat (21), rat (22), human (23)	PRJNA32489
H. brantae	Wild goose (12)	NA
H. canadensis	Human (26), wild goose (27)	PRJNA55359, PRJNA30719 [C]
H. canis	Human (30), cat (31), dog (32)	PRJNA212678
H. cholecystus	Hamster (22)	NA
H. cinaedi	Human, hamster, dog, cat (35, 36), macaque (37)	PRJDA162219 PRJDB88 [C]
H. equorum	Horse (39)	NA
H. fennelliae	Human (41)	PRJDB1096

Table 2.1 Continuation

Gastric		
Helicobacter taxon	**Host**	**Genome Project**
H. pylori	Human (42), non-human primate (43), cat (16), sheep (44), dog (45)	PRJNA57787, PRJNA233 [C]
H. salomonis	Dog (48), human (33),	NA
H. suis	Swine (51), human (52)	PRJNA62531, PRJNA43415
"*H. suncus*"	House musk shrew (54)	NA

Enterohepatic		
Helicobacter taxon	**Host**	**Genome Project**
H. ganmani	Mouse (46), human (47)	NA
H. hepaticus	Mouse, gerbil (49, 50)	PRJNA57737, PRJNA185 [C]
H. macacae	Non-human primate (53)	PRJNA212677
H. marmotae	Prairie dog (55), woodchuck, cat (56)	NA
H. mastomyrinus	Mastomys, mouse (57)	NA
H. mesocricetorum	Hamster (58)	NA
H. muridarum	Mouse, rat (59)	PRJNA224116, PRJNA261711
H. pametenis	Wild bird, swine (60)	PRJNA224116, PRJNA223085
H. pullorum	Human, bird (61-64), mouse (65, 66), rat (67, 68)	PRJNA30075 PRJNA251890 PRJNA251882
H. rodentium	Mouse (69), rat (70)	PRJNA224116, PRJNA234774
H. trogontum	Rat (71)	PRJNA261711
H. typhlonicus	Mouse	PRJNA224116, PRJNA261711

Table 2.1 Continuation

Enterohepatic		
Helicobacter taxon	**Host**	**Genome Project**
H. valdiviensis	Bird (72)	NA
"*H. apodemus*"	Mouse (73)	PRJNA224116, PRJNA261711
''*Candidatus H. bovis*''	Cattle (74)	NA
''*Helicobacter callitrichis*"	Marmoset (75)	NA
''*Helicobacter colifelis*''	Cat (76)	NA
"*Helicobacter jaachi*"	Marmoset (77)	(77)
"*H. magdeburgensis*"	Mouse (78)	PRJNA224116, PRJNA261711
"*Helicobacter muricola*"	Wild mouse (79)	NA
"*H. Winghamensis*"	Human (80)	PRJNA32491

Proposed new species that are currently in processes of validation are represented within quotation marks; ‘Complete genome sequence available; NA: Non-available genome sequence. The genome project number can be accessed in the site: http://www.ncbi.nlm.nih.gov/bioproject/ This table was adapted from (7, 8, 81).

2.2 Clinical Relevance of *Helicobacter* spp.

Helicobacter spp. have been linked to gastrointestinal and hepatobiliary diseases in humans and in animals. Although *H. pylori* colonizes the stomach of more than half of the worldwide human population, the disease development depends on the bacterial virulence components, host susceptibility and environmental factors. *H. pylori* causes chronic gastritis and peptic ulcer disease in 10%-20% of the positive individuals, and gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma in 1%-3% of the hosts. For this reason, in 1994, *H. pylori* was classified as a class I carcinogen (82, 83). Although not very well documented, *H. pylori* has been also associated with gastric diseases in animals such as felines (84) and in non-human primates (85).

Non-pylori GHS and EHS have been isolated from animals with gastric and enterohepatic diseases. Infection of the GHS *H. suis* has been associated with gastritis in pigs, while infection by *H. mustelae*, *H. aurati* and *H. acinonychis* has been related to gastritis and stomach cancer in ferrets, hamsters, and large felines, respectively (8). Concerning EHS pathogenicity in animals, *H. pullorum* may cause enteritis and hepatitis in chickens (86-89), and *H. hepaticus*, *H. bilis* and *H. cholecystus* gastrointestinal and hepatobiliary diseases in rodents (22, 90-93). In mice experimental infections, *H. hepaticus* was shown to induce a chronic inflammation that may develop in hepatitis, consecutive hepatic dysplasia and hepatocellular carcinoma (90, 91). Furthermore, *H. canis* have been associated with intestinal and hepatobiliary disease in cats (31) and dogs (32).

Non-pylori GHS and EHS infections also occur in humans possibly through transmission by contact with infected animals or by meat consumption, as is the case of *H. pullorum* and *H. suis* (8, 94).

In fact, the GHS *H. suis*, *H. salomonis*, *H. felis*, *H. heilmannii* sensu stricto and *H. bizzozeronii* (*i.e. H. heilmannii* sensu lato) shown to colonize the human stomach (Table 2.1). Simultaneous infection of two or more of these species or of a mixture of *H. heilmannii* sensu lato and *H. pylori* have been observed. Although prevalence of *H. heilmannii* sensu lato is very low (0.2% to 6% depending on the geographical area), these organisms have been associated with gastritis, gastric

ulcers and gastric MALT lymphoma in humans (95, 96). Interestingly, the risk of developing tumours seems to be higher among patients infected with *H. heilmannii* sensu lato when compared with those infected by *H. pylori* (97).

EHS have shown to colonize the human intestine and hepatobiliary system and to be associated with gastrointestinal and hepatobiliary diseases. In particular, *H. pullorum* (61, 98, 99), *H. cinaedi* (100), *H. fennelliae* (41, 101), *H. canadensis* (26), *H. winghamensis* (80), *H. canis* (30), and *H. ganmani* (47) have been identified in patients with gastroenteritis, suggesting that infection with these species may be involved in the development of this disease. *H. pullorum*, *H. hepaticus*, *H. bilis* and *H. ganmani* that are bile-tolerant, have been shown to colonize the human hepatobiliary system and are proposed to be associated with chronic liver diseases and hepatic cancer (102-106). Furthermore, *H. bilis* was isolated from the human gallbladder and related with the development of gallbladder cancer (23, 107). Some EHS, such as *H. pullorum* (108, 109) and *H. cinaedi*, (110, 111) were shown to translocate into the systemic circulation and cause bacteraemia. *H. cinaedi* is frequently found in immunocompromised patients (112) and linked to other secondary infections such as endocarditis (113), cellulitis and arthritis (85-87). The observation that the EHS *H. hepaticus*, *H. bilis* and *H. cinaedi* induce intestinal bowel disease (IBD)-like illness in rodent immunodeficient models suggests a possible role of EHS in human IBD aetiology (8, 22, 114). Moreover, *H. pullorum*, *H. fennelliae* and *Helicobacter canadensis* DNA was detected in a significantly higher number of IBD patients than in controls, suggesting an involvement of these species in IBD (115-117).

Nevertheless, it is highly probable that the frequency of infections with non-pylori *Helicobacter* spp. is underestimated in humans due to the fastidious nature of these species, which require nutrient-rich complex media and long incubation times. Furthermore, the phenotypic similarity between member species of the genera *Helicobacter* and *Campylobacter* leads often to a misidentification (118). Therefore, the pathogenic potential of non-pylori *Helicobacter* spp. is undervalued.

2.3 *Helicobacter pylori*

2.3.1 Characteristics and mechanisms of infection

H. pylori presents curved to S-shaped rod cells, with 4 to 8 unipolar or bipolar sheathed flagella (Figure 2.1). Biochemically, *H. pylori* is characterized as urease, catalase, alkaline phosphatase, oxidase positive and nitrate reductase negative. *H. pylori* is resistant to antibiotics such as nalidixic acid and polymyxin B and sensitive to cephalothin (8).

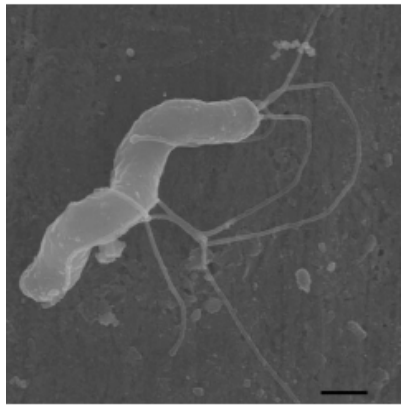

Figure 2.1 Electron micrograph image of *H. pylori*. Bar corresponds to 0.5 μm. Image from (8).

H. pylori is unique in its capacity to colonize the human stomach. The bacterium resists to gastric acidity through the localized concentration of ammonium ions produced by its urease enzyme. Also, the bacterium has evasion ability by moving across the lumen and the gastric mucus layer by means of its flagella (119). Usually *H. pylori* adheres to cells' surface-receptors and does not cross the gastric epithelial barrier, being classified as an extracellular organism. However, *H. pylori* can be internalized by epithelial cells (120) or penetrate the epithelial barrier and be phagocytized (121). In contrast to the majority of bacterial pathogens, which temporarily cause virulent diseases and are then cleared by the immune system, *H. pylori* is able to induce a continuous immune response leading to chronic gastric inflammation (119, 122). In response to *H. pylori*, new

inflammatory cells are constantly being recruited to the infectious foci and produce high levels of ROS and RNS, that although aim to eradicate *H. pylori* also cause DNA damage and tissue injury in the host. In fact, high levels of ROS and RNS are correlated with host histopathological damage and high *H. pylori* concentration in the stomach mucosa (122, 123).

The activation of innate and adaptive immunity by *H. pylori* leads to the recruitment to the stomach of dendritic cells, macrophages, neutrophils, mast cells, T and B cells. *H. pylori* infection results in a mixed Th1 and Th17 cell response, with Th1 cells secreting high levels of INF-γ, and Th17 cells producing IL-17, which enhances IL-8 production. Additionally, Th2 cells were reported to occur at the infection foci but at a smaller extent. More recently, Treg, which suppress effector T cell proliferation and pro-inflammatory cytokine production, were also shown to be involved in the response to *H. pylori* (124).

When adherent to the gastric epithelium, *H. pylori* delivers virulence factors into the cytoplasm of the host cells. Some of the *H. pylori* virulence factors are particularly important for the induction of the host inflammatory response regarding generation of oxidative and nitrosative stresses. For example, the *H. pylori* virulence factors neutrophil activating protein A (NapA) and urease are primarily responsible for the recruitment of neutrophils to the site of infection and for the induction of the neutrophil associated oxidative burst (125, 126). Another *H. pylori* virulence factor is the cytotoxin associated gene A (CagA), which is delivered by the type-IV secretion system to the epithelial cells, that among several cellular functions like cellular growth and motility disruption is responsible for IL-8 production that is a chemoattractant of neutrophils (123). Urease is a potent inducer of the iNOS of macrophages that generate nitrosative stress (127) (Figure 2.2).

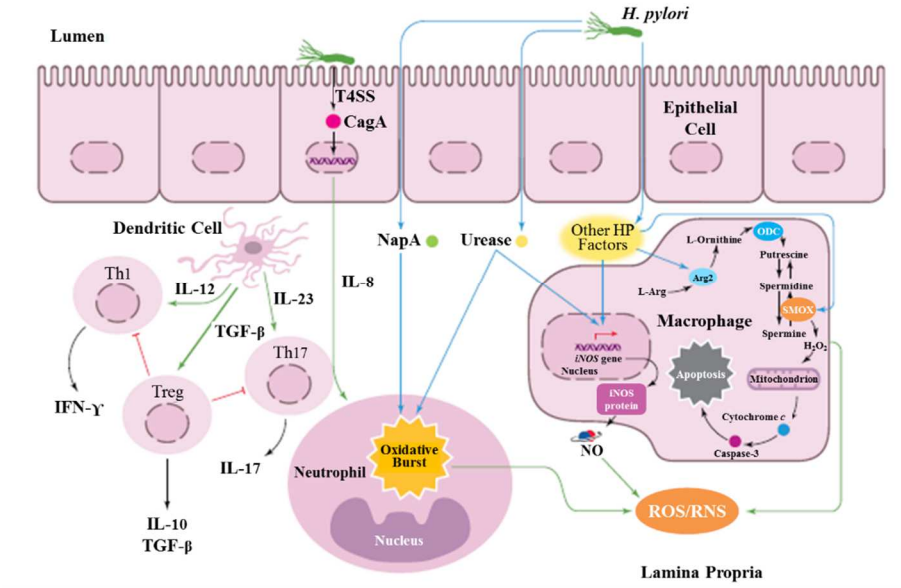

Figure 2.2 Principal mechanisms that promote *H. pylori* continuous inflammation in the host stomach. The virulence *H. pylori* factors CagA, NapA and urease promote neutrophils recruitment and oxidative burst. Macrophage iNOS is induced by urease leading to the production of NO and RNS. Moreover, *H. pylori* induces apoptosis in macrophages via arginase II (Arg2), ornithine decarboxylase (ODC) and spermine oxidase (SMOX) up-regulation. *H. pylori* interaction with dendritic cells results in an increased Treg response and a decreased Th1/Th17 priming. Image from (123).

2.3.2 Protective mechanisms against the host response

H. pylori has been developing strategies to escape the pattern recognition receptors of the innate immune cells, to avoid phagocytic killing, and to promote evasion from adaptive immunity. In particular, the different structure of the lipopolysaccharides (LPS) and flagellin of *H. pylori* contributes to hinder TLR recognition. Similarly to other Gram-negative bacteria, *H. pylori* LPS is composed by a hydrophilic polysaccharide moiety, which contains an O-specific chain, a core region and a hydrophobic lipid A. However, the low degree of phosphorylation and

acylation of the *H. pylori* lipid A has been demonstrated to confer lower biological activity to this LPS. Furthermore, the fucosylated O side chain of *H. pylori* LPS mimics the human Lewis molecules and other blood-group antigens, which contributes to elude TLR4 recognition (128). Also, the specific modification in a conserved N-terminal motif of *H. pylori* flagellin A decreases the level of TLR5 activation (129, 130). Therefore, *H. pylori* LPS is 100 to 10,000 times less reactive than *E. coli* LPS (131) for TLR4 activation, and *H. pylori* flagellin is 1000 times less reactive than *Salmonella enterica* serovar *typhimurium* flagellin for TLR5 activation (130).

To escape phagocytic killing, *H. pylori* interferes with the normal endosomal traffic, fusion and maturation of phagosomes within phagocytes. Several groups reported that *H. pylori* promotes the formation of abnormally big phagosomes containing several bacteria, called “megasomes”, which have impaired interaction with endosomes and therefore lower killing ability (132).

H. pylori causes macrophage apoptosis by induction of enzymes produced by macrophages such as arginase II (Arg2) (133), ornithine decarboxylase (ODC) (134) and spermine oxidase (SMOX) (135) enzymes. Host Arg2 produces L-ornithine and urea from L-arginine, and ODC produces putrescine from L-ornithine that is then converted to the polyamines spermidine and spermine. Once formed, spermine is back-converted to spermidine by SMOX with the release of high levels of hydrogen peroxide that causes mitochondrial membrane depolarization, cytochrome *c* release, activation of caspases pathway and macrophage apoptosis (133, 135) (Figure 2.2).

H. pylori is proposed to evade the adaptive immunity by interacting with dendritic cells, which renders the latter tolerogenic, preferentially inducing Treg differentiation instead of Th1 and Th17 effector responses. Strong Treg and weak T-effector responses favour immune tolerance and persistent *H. pylori* infection (136-138) (Figure 2.2). In addition to its cytotoxic functions, the virulence factor vacuolating cytotoxin A (VacA) inhibits the activation and proliferation of T-cells, by downregulating the surface expression of IL-2 receptor-α and inhibiting the

production of IL-2, which is a cytokine required for T-cell viability and proliferation (124, 139).

H. pylori has been described to control nitrosative stress imposed by the host through the interference with the mammalian iNOS. Arginase enzymes compete with mammalian iNOS for the same L-arginine substrate (140). Gobert and co-workers reported that wild type strains of *H. pylori* inhibit NO production by activated macrophages at physiologic concentrations of L-arginine, and that the inactivation of the gene *rocF*, encoding arginase in *H. pylori*, restored NO production by macrophages and rendered the mutant strain susceptible to macrophage killing (141). Exposure of macrophages to *H. pylori* resulted in the upregulation of host *arg2* and arginase inhibition increased NO production and promoted *H. pylori* killing. In the same study, primary macrophages of *arg2*$^{-/-}$ mice infected with *H. pylori* produced higher levels of iNOS, NO and caused more bacterial killing than the macrophages of wild type mice (142). Altogether, production of bacterial arginase RocF and host arginase Arg2 allow *H. pylori* to evade the immune response by down-regulating eukaryotic NO levels generated by mammalian iNOS.

2.4 *Helicobacter pullorum*

Helicobacter pullorum is an EHS, which was first isolated by Stanley and co-workers in 1994, from the faeces of humans with diarrhoea and from the caeca of healthy broiler chickens and liver and intestines of laying hens suffering from vibrionic hepatitis (61). *H. pullorum* was isolated from other birds such as turkeys, guinea fowls and psittacines (62-64), and more recently it was described to naturally colonize rat (67, 68) and mice (65, 66) (Table 2.1).

Morphologically, *H. pullorum* cells are slightly curved rods with one unsheathed monopolar flagellum (Figure 2.3). The EHS *H. rodentium*, *H. ganmani*, *H. canadensis* and *H. mesocricetorum* also have unsheathed flagella, which is not characteristic in other *Helicobacter* spp. (8). Biochemically, *H. pullorum* is classified as catalase, oxidase and nitrate reductase positive and urease and alkaline

phosphatase negative. In general, *H. pullorum* strains grow in the presence of 1% bile, are sensitive to nalidixic acid and polymyxin B and resistant to the β-lactam antibiotics cephalothin and cefoperazone (61).

The genome of *H. pullorum* is estimated to have between 1.7 Mb and 2.1 Mb and approximately 34% of G + C content (94). Until now, only five draft genome sequences of *H. pullorum* were published (from strains MIT 98- 5489 (143), 229336/12, 229254/12, 229334/12, and 229313/12 (94)), (Table 2.1).

Figure 2.3 Electron micrograph image of *H. pullorum* from (61). Bar corresponds to 1 μm.

H. pullorum seems to adhere to and invade the human intestinal Caco-2 cells through a flagellum-microvillus interaction, which allows the bacterium to anchor onto epithelial cells and penetrate the host cell membrane causing a membrane ruffling effect. In addition, the invasion of *H. pullorum* of Caco-2 cells leads to membrane swelling, microvilli degradation and cellular debris release. By proteomic analysis, the *H. pullorum* type-VI secretion system was suggested to participate in host invasion (144). *H. pullorum* expresses a cytolethal distending toxin (CDT). CDT is a virulence factor produced by many Gram-negative pathogenic bacteria, in which the catalytic subunit, CdtB, is directed to the nucleus of the target cell, promotes DNA damage due to its DNase I-like activity, and causes cytotoxic effects such as cell cycle arrest, and ultimately, cell death. *H. pullorum* CDT promotes multiple cellular damage in intestinal epithelial cell lines, namely: i) formation of nuclear distended giant cells, ii) arrestment of the cell cycle

during the G2/M phase, iii) remodelling of the actin cytoskeleton with the formation of lamellipodia (membrane protrusions (145)), iv) disturbance of host cell adhesion and v) modulation of the cortactin (an F-actin-binding protein (146)) expression (147-149). *H. pullorum* CDT also was shown to induce the expression of genes involved in a Th17 inflammatory response and in cancer development (150). *H. pullorum* LPS presents the highest biological activity within the *Helicobacter* genus and induces nuclear factor-κβ (NF-κβ) activation (151). Furthermore, Varon and co-workers showed that *H. pullorum* cells but not filtered *H. pullorum* supernatants induce IL-8 in gastric and intestinal epithelial cell lines, also through NF-κβ activation, suggesting that the bacteria-host cell interaction is essential for IL-8 production, possible due to the *H. pullorum* LPS attachment to the receptor host cells (152). Recently, Lutay and co-workers described that *H. pullorum* was able to adhere to a J774A.1 macrophage cell line (153); however, so far, no data is available concerning macrophage internalization, susceptibly to macrophage killing and the mechanisms employed by the bacterium against mammalian immune system.

In this work, the characterization of *H. pullorum* macrophage interactions, bacterium susceptibility to nitrosative stress (Chapter V), and enzymatic defences against nitrosative and oxidative stress (Chapter VI) were analysed.

2.5 Treatment and antibiotic resistance

For *H. pylori*, the current first line treatment consist in one of the following therapies: i) a triple therapy that employs a proton pump inhibitor (PPI) and two antibiotics, usually amoxicillin and clarithromycin, used for 10-14 days, ii) a quadruple therapy, that combines a PPI with bismuth and metronidazole and tetracycline, applied for 10-14 days; iii) a sequential therapy that starts with amoxicillin plus a PPI during the first five days and is followed by triple therapy that besides the PPI includes clarithromycin and tinidazole for more five days (154).

Treatment failure is mainly associated with bacterial antibiotic resistance and is one of the major problems associated with *H. pylori*. Although antibiotic resistance patterns vary by country, the general perspective is that *H. pylori* resistance to metronidazole is prevalent while resistance to clarithromycin, amoxicillin and tetracycline is lower (154).

At the molecular level, antibiotic resistance of *H. pylori* has been associated with point mutations. Mutations in the gyrase A (*gyrA*) gene, for example, are associated with fluoroquinolone resistance (155) and mutations in the 23S ribosomal RNA are usually seen in *H. pylori* clarithromycin resistance strains (156).

The antimicrobial action of metronidazole is dependent on its reductive activation by a redox system of the target cell, *i.e.*, nitroimidazole needs to be converted to cytotoxic forms through reduction of the nitro group to exert its antimicrobial effects (157). The oxygen insensitive nitroreductase RdxA, NAD(P)H flavin oxidoreductase FrxA and ferredoxin-like FrxB proteins have been linked with metronidazole activation (158, 159). While it is generally accepted that resistance to metronidazole in *H. pylori* is attributed to mutations in the *rdxA* gene (160, 161), the involvement of *frxA* and *frxB* mutations in metronidazole resistance is still not clear (159, 162). The enzymes nitroreductases will be revised in chapter III of this thesis, and in chapter IV, the new role of the nitroreductase FrxA of *H. pylori* as a defence mechanism against nitrosative injury will be addressed.

Concerning non-pylori *Helicobacter* spp., an optimal treatment regime was not yet developed due to the difficulties in the isolation and culture of these organisms. Therefore, there are no recommended guidelines for performing susceptibility tests nor for the treatment of diagnosed infections with non-pylori *Helicobacter* spp. Nevertheless, the usual treatment currently employed differs between GHS and EHS. For the former, the treatment relies on the conventional antimicrobial therapy used for *H. pylori* (96) and, for the latter, various antibiotics such as penicillin, aminoglycosides, carbapenem and fluoroquinolone used independently or in combination have been used (163-165).

The prevalence of antimicrobial resistance of non-pylori *Helicobacter* spp. have been poorly documented. However, as reported for *H. pylori*, point mutations in the *gyrA* gene of the EHS *H. pullorum* and *H. cinaedi* are associated with

antibiotic resistance. In particular, *H. pullorum* point mutation in codon 84 of the *gyrA* gene, is associated with a resistant phenotype to ciprofloxacin (166) and *H. cinaedi* mutations in codon 84 and/or 88 of *gyrA* gene are associated with fluoroquinolones resistance (163).

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Chapter III

Bacterial defences against oxidative and nitrosative stress

3.1 Bacterial defences against stress

Bacteria employ several strategies to protect against the toxic effects of ROS and RNS, such as the expression of scavenging enzymes, the impairment of ROS and RNS production, and the expression of systems for the maintenance of the redox cellular homeostasis and for the repair of damaged DNA and proteins.

The main scavenging enzymes used by bacteria to counteract the toxic effects of ROS are superoxide dismutases (Sods), superoxide reductases (Sors), catalases and peroxidases.

Sods catalyse the superoxide dismutation into oxygen and hydrogen peroxide (Reaction 3.1). These enzymes are classified in four groups based on their metal co-factor: iron Sod (Fe-Sod), manganese Sod (Mn-Sod), copper-zinc Sod (CuZn-Sod), and nickel Sod (Ni-Sod) (1). The periplasmic CuZn-Sod, encoded by the *sodC* gene, has been reported to play an essential role upon infection, as bacteria having deletions in *sodC* are more susceptible to phagocytic killing and less virulent than their parental strains (2, 3). Bacteria may have three Sods (*e.g. E.coli*), two Sods (*e.g. Staphylococcus aureus*) or one Sod like *H. pylori*. In particular, the Sod of *H. pylori* binds iron (Fe-Sod) and is encoded by the *sodB* gene. While aerobic bacteria use Sod to detoxify superoxide, the anaerobic microorganisms utilize a cytoplasmic superoxide reductase, Sor, which is an iron binding enzyme. Sors catalyse the one-electron reduction of superoxide to give hydrogen peroxide, in the presence of two protons and an external reductant to provide the electron (Reaction 3.2). Unlike Sod, no oxygen is produced, which is an obvious advantage for anaerobic organisms (4).

Reaction 3.1: $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$

Reaction 3.2: $O_2^- + 2H^+ + 1e^- \rightarrow 2H_2O_2$

Bacterial catalases and peroxidases remove the product of Sod and Sor, *i.e.* hydrogen peroxide. Catalases are ubiquitous enzymes that decompose hydrogen peroxide into water and oxygen (Reaction 3.3). The nature of their cofactor classifies catalases into haem and manganese (non-haem) catalases, being the less widespread. Although catalases typically perform the hydrogen peroxide disproportion reaction (Reaction 3.3), some haem catalases have also peroxidatic activity, and are therefore referred as bifunctional catalases or catalase-peroxidases. In general, bacterial species express more than one catalase. Catalase is usually a cytoplasmic protein; however, some studies suggest that bifunctional catalases are secreted to the periplasm in some pathogenic bacteria, including *H. pylori*, enterohaemorrhagic *E. coli* and *Legionella pneumophila* (5). Deletion of catalase genes has been shown to increase the sensitivity of bacterial cells to millimolar levels of hydrogen peroxide (6, 7).

Reaction 3.3 $2H_2O_2 \rightarrow 2H_2O + O_2$

Peroxidases reduce hydrogen peroxide and other toxic peroxides through the oxidation of a co-subtract (RH_2) (Reaction 3.4). The electron donor varies within the peroxidase family of proteins and may be glutathione, thioredoxin, NAD(P)H, and cytochrome *c*. The requirement for a reducing donor, which is not necessary in the case of catalases, is a limiting factor for the efficiency of peroxidases.

Reaction 3.4 $RH_2 + H_2O_2 \rightarrow R + 2H_2O$

Peroxidases are divided into thiol-based peroxidases that include peroxiredoxins (see below, section 3.5), and non-thiol peroxidases, which contain selenium or haem in the catalytic centre. Usually, organisms have several genes encoding peroxidases.

Interestingly, while the wild type *E. coli* cells are not saturated by even millimolar concentrations of hydrogen peroxide (8), the *E. coli* cells lacking the catalases *katG* and *katE* scavenge low concentrations of hydrogen peroxide very effectively but become saturated from 20 μM of hydrogen peroxide. Furthermore, the *E. coli* mutated in both catalases and alkyl hydroperoxide reductase C genes is not able to scavenge hydrogen peroxide (9). Therefore, it has been suggested that peroxidases are usually the primary scavengers at low concentrations of hydrogen peroxide, while catalase activity predominates at higher concentrations of hydrogen peroxide, when peroxidases are saturated due to the slowness of electron delivery and/or are inactivated by hyperoxidation (5, 9).

Regarding nitrosative stress protection, the major bacterial NO detoxifying enzymes are NO reductases (NORs), nitrite reductases, flavodiiron proteins and microbial globins (see below section 3.4).

NORs are membrane-bound iron-containing enzymes that reduce NO to nitrous oxide (Reaction 3.5). NORs are usually active in the denitrification pathway of microorganisms. However, some non-denitrifying bacteria also utilize NOR for the detoxification of NO produced by macrophages (10). NORs have been reported to play an important role in bacterial nitrosative stress protection as the *Neisseria meningitidis* and *Pseudomonas aeruginosa nor* mutants are more susceptible to NO, NO-generating compounds and macrophage killing in comparison to the wild type strains (11-13).

Nitrite reductases are pentahaemic enzymes that promote the six electron reduction of nitrite to ammonium (Reaction 3.6), an important step of the denitrification cycle. Some nitrite reductases also detoxify NO, hydroxylamine (H_2NOH), and nitrous oxide (14). In *E. coli* and *Haemophilus influenza*, the *nrfA* mutant strains are more susceptible to anaerobic NO than the wild type (15, 16). Also, *Campylobacter jejuni* NrfA was suggested to be the periplasmic defence against nitrosative stress while the globin Cgb is proposed to protect the cytoplasmic compartment (17).

Reaction 3.5 $2NO + 2e^- + 2H^+ \rightarrow N_2O + H_2O$

Reaction 3.6 $NO_2^- + 6e^- + 8H^+ \rightarrow NH4^+ + 2H_2O$

Flavodiiron proteins are composed of flavin mononucleotide (FMN) and di-iron catalytic domains. Several members of this family have extra domains, such as flavorubredoxins, which have an additional non-haem mono-iron rubredoxin containing region. Although structurally and mechanistically distinct from the denitrifying NOR, flavodiiron proteins also catalyse the reduction of NO to nitrous oxide (18). The first evidence of NO detoxification by flavodiiron proteins was demonstrated in *E. coli* and attributed to the *norV* and *norW* gene products, which encode the *E. coli* flavorubredoxin or NorV and a cognate oxidoreductase that transfers electrons from NADH to NorV (19, 20). Furthermore, *E. coli* cells lacking *norVW* were shown as attenuated for survival in the presence of macrophages (21). Subsequent studies in other organisms have confirmed the role of flavodiiron proteins in nitrosative stress protection by direct NO consumption (22, 23).

Bacteria also control their intracellular generation of ROS and RNS. Water soluble NAD(P)H quinone oxidoreductases are able two fully reduce quinone molecules, which are components of the respiratory chain. Contrary to the two-electron reduction, the one-electron reduction of oxidized quinone generates semi-quinone radicals, which are able to react with oxygen and form superoxide. Some NAD(P)H quinone oxidoreductases such as the mammalian DT-diaphorase (24) and the bacterial MdaB of *H. pylori* (25), ChrR of *P. putida* (26) and MdaB and WrbA of *P. aeruginosa* (27) were reported to protect cells against oxidative stress through the two-electron transfer reduction of oxidized quinone, avoiding the formation of the dangerously reactive semi-quinone intermediates (28, 29).

Bacteria also employ low molecular weight thiols and thiol-disulfide oxidoreductase systems to maintain the reduced state of the cell. Low molecular weight thiols include cysteine, glutathione, coenzyme A, bacillithiol (*Bacillus* and *Staphylococcus* species) and mycothiol (*Actinomycetes*) (30, 31). The thioredoxin system is composed of thioredoxin reductase (TrxR) and thioredoxin (Trx). In this system, the oxidized TrxR receives electrons from NADPH and reduces Trx, which shuttles the electrons to the terminal thiol containing substrates. The glutaredoxin

system consists of glutathione reductase, glutathione and glutaredoxin. The enzyme glutathione reductase receives electrons from NADPH and reduces the oxidized glutathione that transfers electrons to the oxidized glutaredoxin, and the reduced glutaredoxin transfers electrons to the terminal thiol substrates. Due to its disulfide reductase activity, the two systems present an important role in the defence of bacteria against oxidative and nitrosative stress. However, some bacteria, such as *H. pylori*, lack the glutaredoxin system (32).

Enzymatic mechanisms that repair oxidized DNA and proteins are also operative in bacteria. The methionine sulphoxide reductase (Msr) is an example of an enzymatic system that repairs oxidized proteins. In most bacteria, this enzymatic system is composed by the MsrA and MsrB proteins, which specifically reduce the S- and the R-methionine sulfoxide forms of free and protein-containing oxidized methionine residues, respectively. However, in some bacteria, such as *Streptococcus pneumonia*, *Neisseria gonorrhoeae* and *H. pylori*, the two enzymes are fused in a single protein. The *msrA* mutants of *E. coli* (33, 34) and S*accharomyces cerevisiae* (35) are more susceptible to ROS than the parental strains, and the *E. coli msrA* mutant is also more sensitive to GSNO and nitrite (33). The MsrAB of *N. gonorrhoeae* (36) and *S. pneumonia* (37) mutants have increased susceptibility to ROS. In *S. pneumonia* the *msrAB* mutant has enhanced uptake by macrophages and attenuated virulence in an acute mouse pneumonia model (37).

3.2 *Helicobacter* defences against reactive oxygen and nitrogen species

To protect against the deleterious effects of ROS, *H. pylori* has been reported to encode several enzymes that detoxify ROS such as Sod (38), catalase (39), alkyl hydroperoxide reductase C (AhpC), bacterioferitin comigratory protein (Bcp), and thiol peroxidase (Tpx) (40, 41).

The *H. pylori* strains lacking the Fe-Sod and the bifunctional haem *b* catalase, corresponding to the *sodB* and *katA* mutants, respectively, are significantly more sensitive to hydrogen peroxide than the wild type (6, 42). Though *H. pylori* is a microaerophilic organism and is usually cultivated between 3 and 10% of oxygen

(41), the *sodB* mutant strain was severely inhibited at above 6% of oxygen. Furthermore, the *sodB* gene is up-regulated upon macrophage infection (43). The *H. pylori katA* mutant is hypersensitive to the oxidative burst produced by macrophages (6, 42).

H. pylori defective in alkyl hydroperoxide reductase C (*ahpC*) strains were obtained under low-oxygen conditions and are highly sensitive to oxidative stress generated by organic peroxides and paraquat. At oxygen concentrations where the wild type strain was able to grow (3% to 12%), the viability of the *ahpC* mutant strains was strongly impaired (44).

The *H. pylori* bacterioferitin comigratory protein (*bcp*) mutant exhibits moderate sensitivity to organic peroxides but is not impaired by hydrogen peroxide (40, 41). In addition, the *H. pylori* cells defective in *bcp*, as well as in *ahpC* and in the double *bcp* and *ahpC* mutant, have higher amounts of lipid hydroperoxides, which is the main product of lipid peroxidation. This observation suggests a role for these enzymes in protection from the oxidative unsaturated fatty acids-mediated toxicity (45).

When compared to the wild type, the *H. pylori* thiol peroxidase (*tpx*) mutant strain is markedly sensitive to peroxides and paraquat, and is less able to growth under 10 to 15 % of oxygen (41, 46).

Disruption of any of the above genes in *H. pylori* results in deficiency or attenuation of the ability to colonize mice stomach (25, 40, 43, 46, 47), presenting the *ahpC* and *sodB* mutants the most severe phenotypes (43, 46).

In addition to the enzymes that directly detoxify ROS, *H. pylori* also has other proteins involved in oxidative stress resistance, such as the neutrophil activating protein A (NapA), NADPH quinone oxidoreductase and thioredoxin system.

The *H. pylori* neutrophil activating protein A, NapA, which is involved in the induction of neutrophil oxidative burst (chapter II, section 2.2.1), is a member of the ferritin-like DNA binding proteins from starved cells (Dps) family (48-50).

Dps are iron proteins that form DNA complexes, bind free iron and detoxify hydrogen peroxide coupled to ferrous iron oxidation (51, 52).

The *H. pylori napA* mutant strain is more sensitive to peroxides, paraquat and contains higher intracellular content of free iron and damaged DNA than the wild type. The *napA* mutant does not grow when cultivated among 7 and 15% of oxygen, contrary to the wild type strain (48, 50). Wang and co-workers demonstrated that *H. pylori* NapA binds DNA when the protein is loaded to high levels of iron (107). Furthermore, the *napA* gene is up-regulated in response to the loss of the major oxidative stress resistance genes in *H. pylori* such as *sodB*, *katA*, *ahpC* and *tpx* (49). It was suggested that NapA contributes to the survival of *H. pylori* under oxidative stress conditions due to its ability to bind and protect DNA against oxidation and by binding iron therefore preventing the harmful hydroxyl radical production by Fenton´s reaction, as observed for Dps proteins (48, 50).

H. pylori expresses a NADPH quinone oxidoreductase, encoded by the *mdaB* gene that is up-regulated when *ahpC* and *napA* are absent. *H. pylori mdaB* mutant is moderately sensitive to peroxides and highly susceptible to atmospheric oxygen in comparison to the parental strain. Furthermore, while the wild type strain usually growths at 10% of oxygen, the growth of the mdaB mutant is significantly inhibited under this condition (25). Both the *H. pylori napA* and *mdaB* mutants showed an attenuated ability for mice colonization (44).

The thioredoxin system of *H. pylori* is constituted by thioredoxins Trx1 and Trx2, and a thioredoxin reductase TrxR (53). Trx1 and TrxR form a reductase system for the *H. pylori* AhpC, Bcp and Msr enzymes, where the role of Trx2 is less clear (32). The *H. pylori trx1* mutant is more sensitive to hydrogen peroxide and paraquat than the wild type strain, and grows poorly under 10% of oxygen. On the other side, the *H. pylori trx2* mutant grows very poorly under 10% of oxygen and is hypersensitive to cumene hydroperoxide (41).

H. pylori encodes the universal repair system for oxidatively damaged DNA and proteins. Gene knockouts followed by oxidative stress susceptibility and mice colonization assays revealed that some *H. pylori* DNA repairing proteins such as endonuclease III (Nth), which excises oxidized pyrimidines (54), glycosidase

MutY, which specifically remove adenines paired with 8-oxoguanine (55), MutS that recognizes and repairs 8-oxoguanine (56), and the helicases *recB* and *recN* (57, 58) and endonuclease *ruvC* (59) involved in the DNA homologous recombination process, are important factors for *H. pylori* ROS protection upon colonization. Like for other bacteria, *H. pylori* MsrAB reduces the R isoform of methionine sulfoxide and is up-regulated in response to hydrogen peroxide (60). The *H. pylori msrAB* mutant strain is more susceptible to hydrogen peroxide, paraquat, and 20% of oxygen, and presents a higher level of oxidized proteins and a lower ability to colonize mice (61).

H. pylori also encodes enzymes for NO and RNS detoxification. Saraiva and co-workers reported that *H. pylori* expresses a NO reductase (NorH) enzyme encoded by the *hp0013* gene. NorH inactivation increases the susceptibility of *H. pylori* to the NO donors dipropylenetriamine (DPTA)-NONOate and GSNO, and decreases the NADPH-dependent NO reduction activity of *H. pylori* cells. Accordingly, the recombinant NorH is a NADPH:NO oxidoreductase. The *norH* mutant is more susceptible to macrophage killing and has decreased ability for mice colonization. A phylogenetic analysis revealed that NorH belongs to a novel family of enzymes that are widespread among bacteria (62).

Some of the proteins that protect *H. pylori* from oxidative stress seem also to protect the bacterium from nitrosative injury. In particular, the *H. pylori* peroxiredoxin AhpC was shown to reduce peroxynitrite to nitrite (63). Also, *H. pylori* defective in methionine sulphoxide reductase *msrAB* gene is more sensitive to GSNO than the wild type strain (61). Consistent with these data, *H. pylori msrAB* is up-regulated in response to GSNO and peroxynitrite (61). Trx1 seems also to be involved in *H. pylori* protection against RNS, as growth of *H. pylori trx1* mutant is impaired in the presence of GSNO and nitroprusside. It is proposed that the protective role of *H. pylori* Trx1 against nitrosative stress could be due to its ability in reducing the AhpC and Msr enzymes (41).

As described in chapter I, *H. hepaticus* is an EHS that colonizes rodents and has been associated with gastrointestinal and liver diseases. *H. hepaticus* is a

microaerobic organism that presents an optimal growth at 1% oxygen but still grows well at an oxygen concentration up to 6%.

H. hepaticus enzymatic defences against oxidative stress comprise catalase and the peroxiredoxin alkyl hydroperoxide reductase, encoded by *katA* and *tsaA*, respectively, the DNA binding protein from starved cells (Dps), and NADPH quinone reductase (MdaB).

The *H. hepaticus katA* mutant is more susceptible to hydrogen peroxide than the parental strain, suffering severe DNA fragmentation, and does not survive to 6% of oxygen (7, 64).

The H. hepaticus tsaA mutant is more sensitive to organic peroxides, paraquat, have higher content of lipid hydroperoxides and at 4% and 6% of oxygen grows less than the wild type. However, in the same study, the *tsaA* mutant was shown to be more resistant to hydrogen peroxide and exhibited similar abilities for mice colonization than the wild type strain, which was proposed to be due to a compensatory upregulation of *katA* in the *tsaA* mutant (65).

In *H. hepaticus*, *katA* and *tsaA* genes are induced by hydrogen peroxide and under iron limiting conditions, through the control of the peroxide regulator (PerR) (64). PerR belongs to the ferric uptake regulator (Fur) class of metal-responsive repressor proteins that is present in several bacteria (*e.g. B. subtilis* (66), *S. aureus* (67), and *C. jejuni* (68)). PerR senses hydrogen peroxide and regulates several genes involved in peroxide stress response and haem biosynthesis (69).

In *H. hepaticus*, the *dps* and *mdaB* mutants are more sensitive to peroxides than the wild type strain and have impaired growth at 6% oxygen. Furthermore, the *dps H. hepaticus* mutant present a higher percentage of coccoid or lysed cells when exposed to oxidative stress (70) and the *mdaB* mutant up-expresses the superoxide detoxifying *sod* (71).

Until now, just one enzyme was described to protect the EHS *H. cinaedi* against ROS, the AhpC. Like in other *Helicobacter*, deletion of *ahpC* in *H. cinaedi* decreased the resistance to organic peroxides but increased it to hydrogen peroxide. The mutant strain is more susceptible to macrophage killing and had significantly

reduced colonizing ability of mice. Furthermore, the *ahpC H. cinaedi* mutant displayed a diminished ability to induce a Th1 and Th2 immune response *in vivo*, as the concentration of IgG1 and IgG2a detected in the blood of mice, the serological markers for the Th2 and Th1 responses, respectively, was lower upon mice infection with the mutant than with the wild type strain (72).

3.3 Nitroreductases

Nitroreductases are FMN and flavin adenine dinucleotide (FAD) containing enzymes that reduce the nitro groups (NO_2^-) of a variety of nitrocompounds, using NAD(P)H as reducing agents. Nitroreductases are classified as oxygen insensitive (type I) and oxygen sensitive (type II). The oxygen insensitive or type I nitroreductases catalyse the two electron reduction reaction from NAD(P)H to the nitro group, generating different intermediary products, either in the presence or absence of oxygen. Oxygen sensitive or type II nitroreductases perform a one electron reduction from NAD(P)H to the nitro group generating a nitro anion radical that in the presence of oxygen originates superoxide and regenerates the parental nitro group (73, 74). Figure 3.1 represents the catalytic mechanism used by nitroreductases.

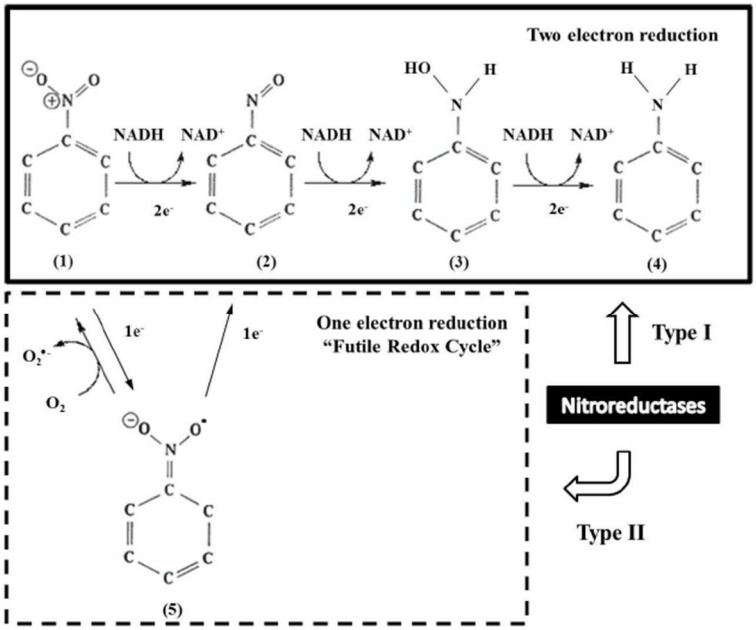

Figure 3.1 The catalytic mechanism of type I and type II nitroreductases. Type I nitroreductases transfer two electrons from NAD(P)H to the substrate (1) resulting in the formation of the nitroso (2), hydroxylamino (3) and the amino group (4). Type II nitroreductases transfer a single electron to the nitro group, forming a nitro anion radical (5), which in the presence of oxygen is reoxidized to the original compound with cogeneration of superoxide in a futile redox cycle or can form the nitroso intermediate by the addition of another electron. Source (75).

Nitroreductases are widely spread in bacteria and found, to a lesser extent, in eukaryotes. Organisms may contain the two types of nitroreductases (75, 76).

Type I nitroreductases are usually homodimers of approximately 20 to 30 kDa and structurally composed of five-stranded β sheets surrounded by helices α. Each monomer contains a FMN prosthetic group, which is bond to a deep pocket at the dimer interface and interacts with residues from both monomers that are conserved among nitroreductases (76, 77). These enzymes are divided in groups A and B, according to the similarity with *E. coli* nitroreductase NfsA and NfsB, respectively. More recently, a group C that contains proteins phylogenetically

related to *S. aureus* nitroreductase NtrA was defined (78, 79). Amino acid sequence similarity among the three groups is very low (less than 10%) (79). Functionally, group A nitroreductases are usually NADPH-dependent whereas group B and C nitroreductases may use both NADH and NADPH as electron donors (76, 79). Most bacteria contain more than one type I nitroreductase gene. *H. pylori*, for example, has two type I nitroreductases, FrxA and RdxA that belong to the B group and present 39% sequence similarity with *E. coli* NfsB (79). Yeast *S. cerevisiae* has also two type I nitroreductases, Frm2 and Hbn1 (80). The only nitroreductase found in mammals is, so far, the type I nitroreductase iodotyrosine deiodinase (YID), which catalyses a reductive deiodination of mono- and diiodotyrosine formed during the biosynthesis of the thyroid hormone thyroxine that prevents the loss of iodide (81, 82). Table 3.1 depicts the distribution and classification of the currently characterized type I nitroreductases.

Table 3.1 Classification of the characterized type I nitroreductases in group A, B and C and respective distribution among different organisms

Organism	**Type I Nitroreductases**			**Refe-**
	Group A	Group B	Group C	**rences**
Bacillus amyloliquefaciens	YwrO			(83)
Bacillus licheniforms		Yfk0		(84)
Bacillus subtilis	NfrA1 (YwcG)			(85, 86)
Clostridium acetobutylicum	NitA, NitB			(87)
Enterobacter cloacae		NR		(88)
Escherichia coli	NfsA	NfsB		(89, 90)
Helicobacter pylori		RdxA, FrxA		(91, 92)
Klebsiella spp. C1		NTR I		(93)
Lactococcus lactis	CinD			(94)
Pseudomonas putida	PnrA	PnrB		(95)
Rhodobacter capsulatus		NprA, NprB		(96)
Salmonella typhimurium	SrnA	Cnr		(97)
Staphylococcus aureus	NfrA		NtrA	(78, 98)
Synechocystis spp. PCC6803	DrgA			(99)
Vibrio fischeri		FRase I		(100)
Vibrio harveyi	Frp			(101)
Homo sapiens	YID			(82)
Saccharomyces cerevisiae			Frm2, Hbn1	(80)

Adapted from (75).

Type II nitroreductases occur in bacteria such as *E. coli* (73) and *Clostridium* strains (102), and their study remains largely unaddressed.

Nitroreductases seem to have several physiological functions. Their main function is the reductive degradation of potentially toxic nitrocompounds, with several nitroreductases degrading a variety of nitroheterocyclic and nitroaromatic substrates. It has been speculated that these compounds are not the nitroreductases original substrates, and that nitroreductases have acquired the capacity to reduce nitroheterocyclic and nitroaromatic compounds released to the environment by human activities. Due to these characteristics, nitroreductases have a biotechnological interest for industrial applications such as for the biodegradation and bioremediation of environmentally toxic nitrocompounds (75, 76).

Nitroreductases also play an essential role in the activation of nitroaromatic antibiotics, such as nitrofurans and nitroimidazoles. The bactericidal activation of these compounds have been attributed to the high cytotoxicity of hydroxylamine intermediates produced upon reduction of their nitro groups by nitroreductases (75, 76). Therefore, bacterial resistance to nitrofurans and nitroimidazoles has been associated with mutations in genes encoding nitroreductases, as is the case of *nfsA* and *nfsB* of *E. coli* (103) and *rdxA* and *frxA* of *H. pylori* (104).

Nitroreductases have also biomedical potential for anticancer therapies. Nitroaromatic prodrugs, such as CB1954 [5-(aziridin-1-yl)-2,4-dinitrobenzamide] are biologically inert compounds that are converted to potent cytotoxic agents when reduced by nitroreductases. In gene or in enzyme-directed prodrug therapy, nitroreductase are delivered specifically to tumour cells before the administration of a nitroaromatic prodrug. The compound can then be activated into a cytotoxic drug at the tumour site and destroys the cancer cells. The *E. coli* NfsA and NfsB enzymes, which activate CB1954, have been the most studied nitroreductases for anticancer prodrug therapy (105); however, other nitroreductases and new prodrugs have also been investigated (106).

Roldán and co-workers suggested that because of the nitroreductases versatility, genetic events such as duplications, lateral gene transfer and mutations have created a set of nitroreductase-like proteins that acquired new physiological roles with or without the loss of the previous activities (76). For example, the

nitroreductase BluB of *Sinorhizobium meliloti* (107) and the mammalian YID (81, 82) have been implicated in the biosynthesis of vitamin B12 and in the reductive dehalogenation of iodinated tyrosine residues during the thyroid hormone synthesis, respectively. An additional role for nitroreductases is the maintenance of cellular metal homeostasis as is the case of *E. coli* NfsA and NfsB and *V. harveyi* FRP, which reduce cromate (Cr^{6+}) to less soluble and toxic Cr^{3+} (108, 109). Moreover, the DrgA nitroreductase of the cyanobacterium *Synechocystis* showed ferric reductase activity when ferric iron was bounded to iron chelator compounds such as EDTA (99). Some nitroreductase-like proteins, such as *V. fischeri* FRaseI and *V. harveyi* FRP are also involved in the bioluminescent process by catalysing the reduction of FMN by NAD(P)H, with the reduced flavin being used as substrate for the luciferase enzyme (100, 101).

Interestingly, some nitroreductases were also shown to be involved in cellular protection against oxidative and nitrosative stress. In *Lactococcus lactis*, mutants of the nitroreductase *cinD* gene are more sensitive to oxidative stress than the wild type, and the purified CinD protein exhibits catalase activity (94).

The *Bacillus subtilis* nitroreductase NfrA1 has catalase activity (85), and the *nfrA1* encoding gene is induced by paraquat and hydrogen peroxide (110). Similarly, the nitroreductase genes *nfsA* of *E. coli*, *snrA* of *S. typhimurium* and *nprA* of *Rhodobacter capsulatus* are induced by paraquat (96, 111, 112).

In *S. aureus*, the *nfrA* transcription is strongly induced by the thiol-specific oxidant diamide and nitrofurantoin, which upon activation generates superoxide, and is slightly induced by high concentrations of hydrogen peroxide. Furthermore, *S. aureus* NfrA exhibits disulfide reductase activity, proposed to contribute to the transfer of electrons to oxidized proteins under oxidative stress conditions (98).

Saraiva and co-workers reported that the *S. aureus* strain defective in another nitroreductase gene, *ntrA*, has greater susceptibility to GSNO killing than the wild type strain, and that *ntrA* is induced by GSNO. Furthermore, *S. aureus* NtrA detoxifies GSNO besides reducing nitrofurans (78). Hence, *S. aureus* NtrA seems to play two different roles *in vivo*: promotion of nitrofuran activation and protection of the cell against nitrosative stress as GSNO reductases (78, 98).

The studies concerning the regulatory mechanisms of nitroreductases are scarce. The *E. coli* NfsA nitroreductase is induced by paraquat through the

superoxide regulator and sensor (SoxRS) system, which responds to superoxide by inducing the expression of scavenger proteins and enzymes that repair damaged proteins and DNA (69). The *E. coli nfsB* expression was shown to be regulated by the multiple antibiotic resistance (MarRA) system, in which the MarA protein binds to a mar box located in the promoter region of the *nfsB* gene (113). The MarRA system controls the response of *E. coli* and other bacteria to different antibiotics and environmental pollutants. MarR is the repressor of the *marRAB* operon that directly interacts with salicylic acid and 2,4-dinitrophenol among other chemicals, leading to MarA synthesis, which directly regulates the transcription of the Mar regulon (114, 115).

In *R. capsulatus*, the expression of the nitroreductase *nprB* gene is proposed to be constitutive, whereas the nitroreductase *nprA* gene expression is inducible by a wide range of nitroaromatic and heterocyclic compounds, including several dinitroaromatics, nitrofuran derivatives, 2-aminofluorene, benzo[a]pyrene, salicylic acid, and paraquat. The identification of two putative *mar/sox* boxes in the possible promoter region of the *nprA* gene and the induction of *nprA* expression by the above mentioned compounds suggest a role of MarRA and SoxRS regulatory systems in the regulation of *R. capsulatus nprA* (96).

3.4 Globins

Globins are a widespread group of enzymes that occur in all kingdoms of life. These molecules are defined by a characteristic globin fold that is composed by helices α surrounding a haem *b* group. Globins are divided in three classes: flavohaemoglobins, single domain haemoglobins and truncated haemoglobins (116). Flavohaemoglobins are sub-divided in type I and type II (117, 118), and truncated haemoglobins in groups I, II and III or N, O and P, respectively (119, 120). Different classes of haemoglobins can co-exist in the same organism, and Table 3.2 summarizes the classification and distribution of the best studied bacterial globins.

Table 3.2 Distribution and number of flavohemoglobins (FHbs), single domain haemoglobins (SHbs) and truncated haemoglobins (TrHbs) in bacteria

Organism	FHbs		SdHbs	TrHbs		
	Type I	Type II		Group I (N)	Group II (O)	Group III (P)
Bacillus subtilis	1				1	
Campylobacter jejuni			1			1
Escherichia coli	1					
Helicobacter hepaticus						1
Mycobacterium avium		1		1	1	1
Mycobacterium bovis		1		1	1	
Mycobacterium leprae					1	
Mycobacterium smegmatis	1	1		1	1	
Mycobacterium tuberculosis		1		1	1	
Pseudoalteromonas haloplanktis	1			1	2	
Pseudomonas aeruginosa	1		1			
Salmonella typhimurium	1					
Staphylococcus aureus	1				1	
Synechococcus spp. PCC 7002				1		
Vibrio cholerae	1		1			
Vitreoscilla stercoraria			1			

Data from (121, 122).

3.4.1 Flavohaemoglobins

The flavohaemoglobins are present in bacteria, yeast and fungi. These enzymes present a unique two domain structure composed of an N-terminal haemoglobin domain fused with a highly conserved C-terminal oxidoreductase domain that binds NADPH and FAD. The N-terminal domain of flavohemoglobins has the classical 3-on-3 haemoglobin fold surrounding the haem centre (Figure 3.2) (123, 124).

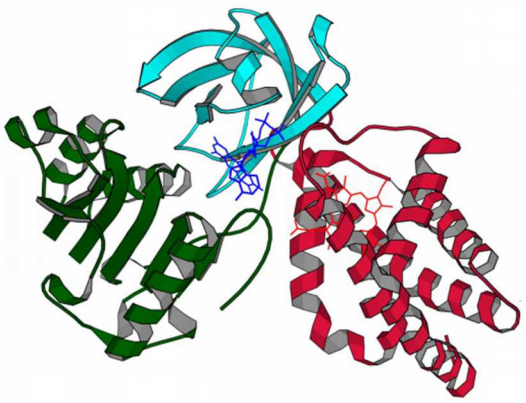

Figure 3.2 Structure of *E. coli* flavohaemoglobin. The N-terminal classical globin domain of flavohaemoglobin is represented in red, while the FAD and the NADPH-binding modules of the C-terminal oxidoreductase domain are represented in cyan and green, respectively. Adapted from (125).

Type I flavohaemoglobins have been shown to protect several bacteria against nitrosative stress by direct NO consumption, including *E. coli* (126), *V. cholerae* (127), *P. aeruginosa* (128), *S. aureus* (129, 130), *B. subtilis* (131), and *S. typhimurium* (132).

In aerobic conditions, type I flavohaemoglobins catalyse the conversion of NO to nitrate, through a NO dioxygenase or a NO denitroxylase mechanism (Reaction 3.7).

Reaction 3.7 $2NO + 2O_2 + NADPH \rightarrow 2NO_3^- + NADP^+ + H^+$

In the dioxygenase mechanism, the reaction starts with the binding of the oxygen to the ferrous haem and the formation of a ferrous-oxy complex, which reacts with NO to form a transient ferrous-$ONOO^-$ intermediate that undergoes a rapid isomerization and releases nitrate with the concomitant oxidation of the ferrous to ferric haem. The ferrous haem is then restored by transfer of an electron from NADH to FAD, and to the ferric haem.

In the denitroxylase mechanism, NO first binds ferrous haem to form a ferrous-nitrosyl species, followed by the electron transfer from the ferrous haem of the ferrous nitrosyl species to generate a ferric-nitroxyl species, which in turn reacts with oxygen to form nitrate. The oxidoreductase domain supports the one-electron reduction of the ferric to ferrous haem, thus completing the catalytic cycle. The higher affinity of ferrous flavohaemoglobin to NO when compared with oxygen and the fact that bacteria face high concentrations of NO generated by macrophages in low oxygen environments suggests that the denitroxylase mechanism predominates over the dioxygenase mechanism under physiological conditions (123, 129).

Under anaerobic conditions, type I flavohaemoglobins convert NO to nitrous oxide, but this reaction occurs at approximately 1% of the aerobic reaction, which suggests that flavohaemoglobins operate mainly under aerobic conditions (133).

Under anaerobic conditions, flavohaemoglobins also reduce hydroperoxides (*e.g.* t-butyl hydroperoxide, cumene hydroperoxide and linoleic acid hydroperoxide), and may therefore protect the cells from these toxic peroxides (134).

The expression of type I flavohaemoglobin is increased by: i) NO and NO related compounds; ii) the decrease of iron (132, 135); iii) limitation of oxygen (129); iv) by paraquat; and v) in the stationary cell growth phase (136). Its regulation involves several transcription factors that may vary within bacteria. For example, the NO sensitive repressor (NsrR) regulates the flavohaemoglobin response to NO donors, acting as repressor in *Salmonella typhimurium* (132), *B. subtilis* (137) and *E. coli* (138), under aerobic and anaerobic conditions. NsrR is a NO sensor that contains a 2Fe-2S cluster whose nitrosylation results in the loss of its DNA binding activity and de-repression of the flavohaemoglobin transcription.

The global fumarate nitrate reduction (FNR) transcription factor also regulates the expression of the flavohaemoglobin gene (*hmp*) in *E. coli* (135). FNR is able to sense oxygen and NO via its labile 4Fe-4S cluster. FNR becames inactive in response to the increase in oxygen levels through oxidation of the 4Fe-4S to 2Fe-2S and the loss of the cluster. As the oxygen levels decrease, the FNR cluster is loaded again and the protein is activated (139). Under anaerobic conditions, when FNR is in its holo form, the *E. coli hmp* expression is repressed (135) but when the bacteria are exposed to NO under anaerobic conditions, the 4Fe-4S became nitrosylated leading to a decrease in the affinity of FNR to the *hmp* promoter and to a de-repression of the *hmp* transcription (140).

More recently, a new type of flavohaemoglobins have been identified in *Mycobacteria* and in other *Actinomycetes*. These proteins were classified as type II flavohaemoglobins and, unlike the conventional type I, its haem group displays an hexa-coordinated form in both ferric and ferrous states, and the C-domain lacks several of the conserved residues involved in FAD and NADH binding in type I flavohaemoglobins (117). Type II flavohaemoglobins may also co-exist with truncated haemoglobins and type I flavohaemoglobins (Table 3.2). While type I *Mycobacteria* flavohaemoglobin detoxifies NO (118), the type II flavohaemoglobin do not performs NO detoxification; instead, it oxidizes D-lactate, which may accumulate during membrane lipid peroxidation under oxidative stress conditions, by transferring electrons from D-lactate to the haem via FAD. *In vitro*, the type II flavohaemoglobin gene of *M. tuberculosis* is up-regulated by hydrogen peroxide and *in vivo* during macrophage infection (141).

3.4.2 Single domain haemoglobins

Single domain haemoglobins exhibit the classical 3-on-3 α-helical myoglobin fold, characterized by a haem group surrounded by eight helices α (Figure 3.3) (116). This class of enzymes is present in bacteria such as *C. jejuni*, *Pseudomonas aeruginosa* and *Vibrio cholerae* (Table 3.2), and in eukaryotes (142). The structure and amino acid sequence of bacterial single domain haemoglobins are

closely related to the 3-on-3 eukaryotic globins (*e.g*. myoglobin, neuroglobin and cytoglobin) (121).

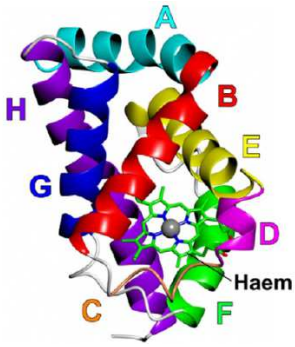

Figure 3.3 Structure of the single domain haemoglobin Cgb of *C. jejuni*. The figure depicts the 3-on-3 α-helical fold of Cgb surrounding the haem cofactor. Helices are represented in different colours and labelled from A to H. Image from (143).

The role of bacterial single domain haemoglobins have been studied in *Vitreoscilla* and in *C. jejuni*.

Vitreoscilla is a Gram-negative obligate aerobic bacterium that grows in low oxygen environments (144), and has a unique haemoglobin belonging to the single domain globin class that was named Vhb (or Vgb). The expression of the *vhb* gene in its natural host (145) and its heterologous expression in *E. coli*, when *vhb* is cloned together with its natural promoter (146), is strongly induced by hypoxic conditions through the global transcription regulator Fnr (147). In addition, the heterologous expression of *Vitreoscilla vhb* in *E. coli* and in other organisms resulted in improved cell growth and protein production under microaerobic conditions (148, 149). These observations had led to suggest a role for *Vitreoscilla* Vhb in oxygen binding (particularly at low concentrations) and its delivery to the terminal respiratory oxidase enzymes to enhance ATP production, which would enable *Vitreoscilla* to survive under hypoxic conditions (150). Alternatively, it was suggested that Vhb may act as a terminal oxidase itself (148).

The expression of *Vitreoscilla* Vhb in several hosts has become a widely used biotechnological strategy to enhance the production of a variety of bio-products,

stimulate bioremediation, and increase the growth yield and survival of engineered organisms (151).

In addition to the role in oxygen metabolism, other protective functions have been proposed for *Vitreoscilla* Vhb. In particular, it was reported that the heterologous expression of Vhb in *Enterobacter aerogenes* reduces the hydrogen peroxide toxicity (152), and that *Vitreoscilla* Vhb possesses peroxidase activity (153). The heterologous expression of *Vitreoscilla vhb* in *E. coli* resulted in the induction of the antioxidant genes *katG* (catalase G) and *sodA* (superoxide dismutase A), and conferred significant protection to *E. coli* from oxidative stress. This protective effect of *Vitreoscilla* Vhb was mediated through the *E. coli* oxidative stress regulator OxyR and Fnr (154). OxyR is a transcriptional regulator that senses hydrogen peroxide through a sensory cysteine residue and regulates the response of several bacteria to oxidative stress (69). *Vitreoscilla* Vhb was shown to directly bind to *E. coli* OxyR, and to promote OxyR oxidation, which may had result in the up-regulation of *katG*, *sodA* and possibly of other genes that are part of the OxyR regulon (154).

A role for Vhb in nitrosative stress protection has also been proposed. A chimeric protein comprising Vhb fused with a flavoreductase domain of the *Ralstonia eutropha* FHb was described to consume NO and to provide *E. coli* protection against nitrosative stress (155). It was proposed that a flavoreductase (NADH-methemoglobin reductase) that co-purifies with Vhb may act as the reducing partner (156), even though, the overall mechanism of NO detoxification by *Vitreoscilla* remains unknown.

In contrast to *Vitreoscilla* Vhb, there is no evidence that the single domain globin Cgb of the microaerophilic *C. jejuni* plays a role in oxygen delivery. The *C. jejuni cgb* mutant strain exhibited markedly inhibition of the aerobic respiration and hypersensitivity to NO and other nitrosating agents (157-159). *Cgb* is strongly up-regulated by nitrosative stress through NssR as the disruption of *nssR* abolished the inducible expression of *cgb*. The nitrosative stress sensing regulator (NssR) belongs to the cAMP receptor protein (Crp)-Fnr superfamily of transcription regulators and is commonly encoded in ε-proteobacterial genomes (160). Furthermore, *C. jejuni*

Cgb has been shown to detoxify NO (161), but its partner reductase remains unknown, and it is speculated that Cgb may associate with host reductases or use bacterial small molecules (*e.g.* GSH) as a source of electrons for NO (162). Therefore, it is well established that resistance against nitrosative stress is linked to the presence of Cgb in *C. jejuni*.

3.4.3 Truncated haemoglobins

The truncated haemoglobins are widely distributed in bacteria, unicellular eukaryotes and plants (163). As their name indicates, truncated haemoglobins are 20 to 40 residues shorter than single domain haemoglobins. Furthermore, truncated haemoglobins present a distinctive 2-on-2 α-helical fold in which the conserved helix pairs B/E and G/H are arranged in an antiparallel way surrounding and protecting the haem group from the solvent (Figure 3.4).

Sequence identity among the truncated haemoglobins belonging to groups I, II and III is very low (~ 20% identity). The main structural differences among these groups are the A-helix that can be either very short or absent (as in group III) and the variation in the length of F and H-helices (Figure 3.4) (119, 120).

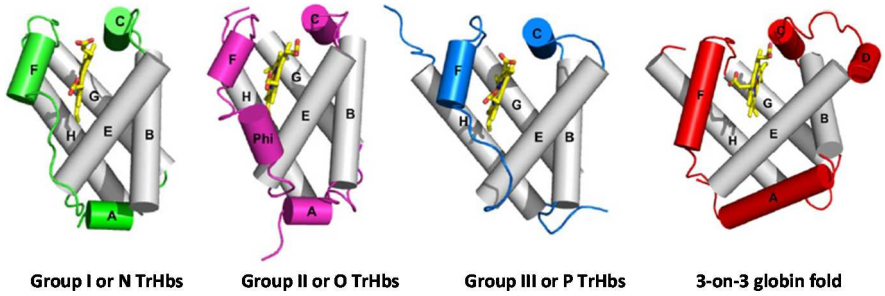

Figure 3.4 Structure of the three classes of truncated haemoglobins. Truncated haemoglobins (TrHbs) of groups I (N), II (O) and III (P) are represented by *Paramecium caudatum* HbN, *M. tuberculosis* HbO and *C. jejuni* Ctb, respectively. For comparative purposes, the structure of 3-on-3 haemoglobins is represented by

the sperm whale globin. Helices are shown as cylinders and labelled. The helices structurally conserved within 3-on-3 and 2-on-2 folds are shown in grey. The haem is shown in a stick representation. Adapted from (164).

The role of the truncated haemoglobins belonging to group I and II was studied in the cyanobacterium *Synechococcus* spp. PCC 7002, in *Mycobacterium* species, and in *Pseudoalteromonas haloplanktis*.

The *Synechococcus* spp. PCC 7002 haemoglobin (*glbN*) is encoded by GlbN that belongs to the group I truncated haemoglobins. High levels of nitrate, used as the nitrogen source, and exposure to NO were better tolerated by the wild type strain than by the *glbN* mutant. Furthermore, the cellular contents of ROS and RNS were elevated in the *glbN* mutant under nitrosative stress conditions and were significantly attenuated upon GlbN overproduction. Therefore, the authors proposed that GlbN protects cells from RNS that could be naturally encountered during growth on nitrate or under denitrifying conditions (165).

M. tuberculosis and *M. bovis* have two truncated haemoglobin encoding genes, *glbN* and *glbO*, corresponding to the proteins HbN and HbO, which belong to the truncated haemoglobin group I and group II, respectively (119) (Table 3.2). *M. tuberculosis* truncated haemoglobin HbN presents NO activity. Furthermore, the over-expression of *M. tuberculosis glbN* affords protection against NO toxicity in an *E. coli hmp* mutant (166). In *S. typhimurium hmp* deficient cells expressing *glbN* and *glbO*, the *glbN* gene but not *glbO* improved bacterial survival within activated macrophages. Under aerobic conditions, the NO uptake activity of a *S. typhimurium hmp* mutant was significantly improved upon expression of *M. tuberculosis glbN* and *glbO*. While under low oxygen levels, HbN efficiently remove NO, the HbO does not, suggesting a predominant role of *M. tuberculosis* HbN over HbO in NO detoxification (167).

M. bovis HbN detoxifies NO into nitrate, and the *glbN* mutant strain does not metabolize NO and has decreased NO respiration rates (119).

M. leprae group II truncated haemoglobin (HbO), the only globin apparently present in the genome, was shown to be involved in hydrogen peroxide, NO and peroxynitrite scavenging (168-170). Since it is apparently the only globin in *M. leprae*, it has been suggested that the bacterium has lost HbN during evolution and HbO may have acquired the ability to detoxify NO (169).

P. haloplanktis Ph-2/2HbO, which is encoded by *PSHAa0030* gene and is one of the group II truncated haemoglobins of the marine cold-adapted psychrophile *P. haloplanktis*, was shown to catalyse peroxynitrite isomerization *in vitro*. In addition, Ph-2/2HbO was shown to confer *P. haloplanktis* resistance against oxidative and nitrosative stresses and to protect the growth and cellular respiration of an *E. coli hmp* mutant under nitrosative stress conditions (171, 172).

Among group III, the truncated haemoglobins from *C. jejuni* (Ctb) and *H. hepaticus* (HbP) (173) are the best biochemically characterized proteins, but their physiological role was so far only investigated for Ctb.

Although its expression is elevated when exposed to NO and RNS, via the action of the NO-responsive regulator NssR (158), mutation of the *ctb* gene in *C. jejuni* does not confer an NO-sensitive phenotype (174). The *C. jejuni ctb* mutant has a slower growth rate during the stationary phase under microaerobic conditions when compared with the wild type strain, suggesting a role for Ctb in oxygen transfer. However, under oxygen-limited conditions, the *C. jejuni ctb* mutant showed similar growth levels than the parental strain (174).

3.5 Peroxiredoxins

Peroxiredoxins are thiol-dependent peroxidases that detoxify hydrogen peroxide, peroxynitrite and organic peroxides.

Peroxiredoxins are characterized by a conserved thioredoxin fold consisting of seven strands β and five helices α. Their active centre is structurally conserved, containing a highly conserved catalytic cysteine residue referred as the peroxidatic cysteine (C_P). When present, a second cysteine residue which is involved in catalysis and termed resolving cysteine (C_R), is localized at the C-

terminal helix or in other distinct positions (in helix α2, α3, α5 or between β1and β2 strands). In general, peroxiredoxins can exist as monomers; dimers or assemble into active decameric and dodecameric structures (175, 176). All peroxiredoxins share a common catalytic cycle, in which the thiolate group of the C_P attacks a peroxide substrate. This cysteine is then oxidized to sulfenic acid with the release of the corresponding water or alcohol when hydrogen peroxide or larger peroxides are used as substrates, respectively. Subsequently, the oxidized C_P forms a disulfide linkage with the C_R and the disulfide is reduced by a thiol protein, regenerating the C_P and C_R residues (Figure 3.5) (175, 177).

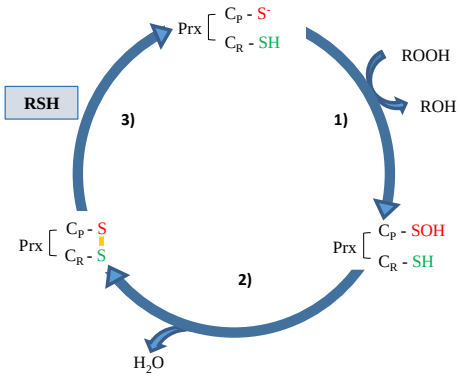

Figure 3.5 Catalytic cycle of peroxiredoxins. The peroxidatic cysteine thiolate (C_P-S^-) attacks a peroxide substrate (ROOH) and is oxidized to a sulfenic acid (C_P-SOH) (1). The resolving cysteine (C_R-SH) forms a disulfide with the C_P-SOH (2), and the disulfide is reduced by a thiol protein (RSH) regenerating the C_P-S^- and the C_R-SH (3). Image adapted from (175, 177).

Peroxiredoxins were originally classified as typical 2-Cys, atypical 2-Cys and 1-Cys according to the number and location of the catalytic cysteines. In general, the 2-Cys peroxiredoxins are homodimeric enzymes that contain one C_P and one C_R per subunit. In the family of the 2-Cys peroxiredoxins, the C_R is found in the C-terminal helix and forms an intersubunit disulfide with the C_P of the other subunit. In the atypical 2-Cys peroxiredoxins, the C_R is not located in the C-terminal helix and each C_R reacts with the C_P of the same subunit to form an

intrasubunit disulfide. Given the absence of the C_R, the 1-Cys peroxiredoxins form a disulfide with a cysteine of another thiol protein such as thioredoxin, glutaredoxin or glutathione (175, 177, 178).

Since this classification system only informs about the catalytic mechanism of each enzyme, a more recent classification of peroxiredoxins based on sequence similarities was proposed. This new classification divides peroxiredoxins into: i) peroxiredoxin 1/alkyl hydroperoxide reductase C (Prx1/AhpC); ii) bacterioferitin/peroxiredoxin Q (Bcp/PrxQ); iii) thiol peroxidase (Tpx); iv) peroxiredoxin 6 (Prx6); v) peroxiredoxin 5 (Prx5); and vi) alkyl hydroperoxide reductase E (AhpE) (179). Most organisms have several classes of peroxiredoxins coexisting in the genome. Table 3.3 gives examples of the distribution of the six subfamilies of peroxiredoxins in organisms from different kingdoms of life.

Table 3.3 Distribution, classification, and number of peroxiredoxins present in different organisms

Organism	Peroxiredoxin subfamilies						References
	Prx1/AhpC	Bcp/PrxQ	Tpx	Prx6	Prx5	AhpE	
Bacteroides fragilis	1	1	1				(180, 181)
Brucella abortus	1	1			1		(182)
Campylobacter jejuni	1	1	1				(183)
Desulfovibrio vulgaris Hildenborough	1	1	1				(184, 185)
Enterococcus faecalis	1		1				(186),
Escherichia coli	1	1	1				(175)
Francisella tularensis	1	1			1		(187)
Helicobacter pylori	1	1	1				(41)
Mycobacterium tuberculosis	1	2	1			1	(188)
Porphyromonas gingivalis	1	1	1				(189)
Staphylococcus aureus	1	1	1				(190, 191)
Streptococcus pyogenes	1						(192)
Giardia intestinalis	2						(193)
Homo sapiens	4			1	1		(175)
Saccharomyces cerevisiae	2	1		1	1		(194)
Trypanosoma cruzi	2						(195)
Xylella fastidiosa	1	1					(196)

The members of the Prx1/AhpC subfamily are typical 2-Cys proteins and are widely distributed in all kingdoms of life. This subfamily is the best studied one and includes the yeast thiol-specific antioxidant (TSA) proteins, several plant peroxiredoxins, the bacterial AhpC (named as such due to the alkyl hydroperoxide reductase activity demonstrated by the first bacterial enzyme found of this class) and the human PrxI, PrxII, PrxIII and PrxIV (175, 176). The members of this subfamily are typically dimeric proteins and undergo a redox-sensitive dimer-decamer or a less usual dimer-dodecamer transition, in which the oxidized protein favours catalytic dimers while the reduced AhpC dimers preferentially assemble in a doughnut-like structure. The main structural difference of the Prx1/AhpC compared with other peroxiredoxin subfamilies is the C_R present at the C terminal helix and a C-terminal extension of 50 to 80 residues (175). Some bacteria such as

E. coli (197) and *S. typhimurium* (198) have a cognate flavoreductase, AhpF, that specifically reduces AhpC. In other bacteria, like *H. pylori* (199), AhpC is also present but its reducing partner, AhpF, is absent. Instead, the latter bacterium uses the thioredoxin/thioredoxin reductase system, or other thiol proteins, to reduce AhpC. The Prx1/AhpC peroxiredoxins play an essential role in protecting organisms against oxidative and nitrosative damage by directly detoxifying toxic peroxides. These enzymes are able to detoxify a wide peroxides such as hydrogen peroxide, t-butyl hydroperoxide, cumene hydroperoxide and lipoic acid hydroperoxide, but with different substrate affinities that depend on the organism (200). Furthermore, AhpC from the *S. typhimurium*, *M. tuberculosis*, and *H. pylori* (63), and Prx1/AhpC members from higher organisms such as *S. cerevisiae* Tsa1 and Tsa2 (201), *Plasmodium falciparum* TPx1 (202), *Trypanosoma brucei* and *Trypanosoma cruzi* TXNPx (203) and human Prx2 (204) efficiently reduce peroxynitrite. Phenotypic studies reported that the *Listeria monocytogenes* (205), *Porphyromonas gingivalis* (206) and *Brucella abortus* (182) *ahpC* mutants are more sensitive to hydrogen peroxide relatively to the wild type strain. On the contrary, the *ahpC* deletion mutants of bacteria such as *S. aureus* (207), *H. hepaticus* (65), *H. cinaedi* (72), *M. tuberculosis* (208), *C. jejuni* (209), *Streptococcus pyogenes* (192), *Streptococcus mutans* (210) and *E. coli* (9) showed little or no sensitivity to hydrogen peroxide. However, it was shown that this lack of susceptibility is due to a compensatory upregulation of catalase levels, which detoxifies hydrogen peroxide in the absence of AhpC.

Several bacterial strains with *ahpC* inactivated gene are sensitive to organic peroxides. In particular, *C. jejuni* (209), *H. cinaedi* (72), *H. hepaticus* (65), *H. pylori* (44) and *S. typhimurium* (211) *ahpC* mutants have diminished resistance to cumene hydroperoxide. *H. cinaedi* (72), *H. hepaticus* (65) and *H. pylori* (44) *ahpC* mutants have increased susceptibility to t-butyl hydroperoxide. Hence, AhpC seems to have a role as a scavenger of organic peroxides. Also, an accumulation of lipid hydroperoxides was observed in the *H. hepaticus tsaA* (65) and *H. pylori ahpC* (45) mutant strains.

In bacteria such as *L. monocytogenes* (205), *M. tuberculosis*, *M. smegmatis* (212), *B. abortus* (182), and *Francisella tularensis* (213), the *ahpC* mutation diminuished

the resistence to peroxynitrite or to peroxynitrite generators. Furthermore, the *S. typhimurium ahpC* mutant showed increased susceptibility to peroxynitrite, GSNO and sodium nitrite, a defect that was complemented by *M. tuberculosis* AhpC (211). Also, the deletion in *S. cerevisiae* of *tsa1* and *tsa2* genes rendered the strain hypersensitive to peroxynitrite and sodium nitroprusside (214).

AhpC seems to be an important mechanism for bacterial survival within macrophages. In particular, the *ahpC* mutants of *H. cinaedi*, *M. tuberculosis* and *F. tularensis* have decreased survival upon macrophage infection (72, 212, 213). Furthermore, *S. typhimurium* (215, 216) and *E. coli* (217) *ahpC* and *ahpF* expression is induced upon intracellular growth in mouse macrophages and in response to phagocytosis by human neutrophils, respectively.

AhpC was also shown to be an important virulence factor upon mice infection, as the *ahpC* mutants of *H. pylori* (46) presented a severe deficiency for mice colonization. Moreover, the mutants of the *ahpC* homologous genes in *H. cinaedi* (72) and *L. monocytogenes* (205) have diminished virulence.

The regulatory mechanism of *ahpC* varies among organisms. *H. pylori* seems to lack the homologs of the major peroxide regulatory systems such as OxyR and PerR (200), and the regulation of *ahpC* is linked to the post-transcription aconitaseB, which is a 4Fe-4S protein. AconitaseB cluster is oxidized under high oxygen levels or disassembled under low iron conditions, rendering apo-aconitaseB that is able to bind to the transcript of *ahpC*, and provides RNA stability and subsequently increase of *ahpC* expression (218). In *E. coli* (219), *S. typhimurium* (215), *P. aeruginosa (220)*, *Mycobacteria* (221), *P. gingivalis* (222), *Bacteroides fragilis* (223) and *Streptomyces coelicolor* (224) the *ahpC* gene is regulated by OxyR in response to hydrogen peroxide. PerR has also been identified as the regulator of peroxide-inducible *ahpC* expression in *B. subtilis* (66), *C. jejuni* (68), *H. hepaticus* (64), and *S. aureus* (191).

C. jejuni oxidative stress regulator (CosR) also shown to positively control the expression of *ahpC*. CosR is present in other ε-Proteobacteria such as *H. pylori*, *H. hepaticus* and *Wolinella succinogenes*. In response to paraquat, the level of CosR

decreases together with the expression of *ahpC*, which suggests that AhpC may contribute to the detoxification of peroxides and not of superoxide (225).

The hydrogen peroxide regulator (HypR) is a new oxidative stress regulator that was isolated and characterized in the enteric bacterium *Enterococcus faecalis*. HypR is a member of the LysR family of positive transcriptional regulators and was shown to be involved in the activation of *E. faecalis ahpCF* in response to hydrogen peroxide (226).

Some eukaryotic Prx1/AhpC enzymes shown to be inactivated at high intracellular hydrogen peroxide levels by hyperoxidation of the C_P, which is then reversed by sulfiredoxins and sestrins reduction. However, and contrary to eukaryotes, the majority of the bacterial peroxiredoxins are highly resistant to inactivation by hyperoxidation, even at millimolar peroxide concentrations, which permits bacteria to survive high levels of ROS and RNS produced by mammalian macrophages (175, 227, 228). Finally, some Prx1/AhpC enzymes were reported to function under stress conditions as molecular chaperones in organisms from different kingdoms of life, as is the case of *H. pylori* AhpC, cyanobacterial *Anabaena* 2-Cys peroxiredoxin alr4641 and *P. aeruginosa* PaPrx (229).

Although the majority of the Bcp/PrxQ members occur in bacteria, these proteins are also present in archaea and eukaryotes. Bcp was originally identified in *E. coli* as a protein that co-migrated with bacterioferritin in SDS gels (230). The Bcp/PrxQ is structurally and functionally the most diverse peroxiredoxin subfamily. Known members vary in its oligomeric state, existing as monomers or dimers, as well as in the presence and location of the C_R, having 1-Cys or 2-Cys mechanisms (175). The Bcp/PrxQ proteins are able to detoxify a wide range of peroxides, but with activity rates lower than the bacterial AhpC proteins (231). Interestingly, the *E. coli* Bcp protein demonstrated a higher affinity for reducing linoleic acid hydroperoxide (Km = 10 μM) than for hydrogen peroxide (Km = 50 μM) or t-butyl hydroperoxide (Km = 40 μM) (232). Similarity, the *H. pylori* Bcp showed preference for linoleic acid hydroperoxide, suggesting that the reduction of fatty acids hydroperoxides might be one of the main roles of Bcp (45). In plants, *Xylella fastidiosa* PrxQ was reported to detoxify peroxynitrite (196), but no similar

function was so far observed in bacterial Bcps. Phenotypical studies showed that the *Burkholderia cenocepacia* (233), *E. coli* (232) and *P. gingivalis* (234) *bcp* mutants are more sensitive to hydrogen peroxide than the wild type strains. Furthermore, the *E. coli bcp* mutant is hypersensitive to organic peroxides (232). The expression of the *bcp* gene of *P. gingivalis* (234) and *B. fragilis* (181) restored the hydrogen peroxide resistance of an *E. coli bcp* defective mutant. In *C. jejuni*, the single *bcp* and *tpx* mutants had no major alteration in their phenotype when exposed to peroxides, superoxide, or nitrosative stress agents. Nevertheless, a double mutation in the *bcp* and *tpx* genes rendered the strain hypersensitive to all of these agents, suggesting that Bcp and Tpx are partially redundant in this organism (183). In *H. pylori*, the *bcp* mutant was only slightly sensitive to oxidative stress (40). Yet, Bcp was shown to protect *H. pylori* from lipid peroxidation and to contribute to virulence during mice infection (see above section 3.2) (40).

Although the studies of *bcp* regulation are scarce, in *S. aureus*, PerR was shown to induce the transcription of *bcp* in response to hydrogen peroxide (191).

The Tpx like proteins are only present in bacteria. This subfamily was named as such for its first discovered member thiol peroxidase of *E. coli*. Relatively to the other peroxiredoxin subfamilies, Tpxs have an additional N-terminal β hairpin loop in their structure. Tpxs are obligate dimers, with almost all identified Tpx functioning as 2-Cys and about 1% as 1-Cys peroxiredoxins (175). The Tpx family members have a broad substrate specificity (235, 236) that varies among organisms. For example, *E. coli* Tpx reduces hydrogen peroxide, t-butyl hydroperoxide, cumene hydroperoxide and linoleic acid hydroperoxide more efficiently than AhpC and shows a distinct preference for cumene hydroperoxide (Km = 9 μM) over hydrogen peroxide (Km = 1.7 mM) (236). *M. tuberculosis* Tpx also presents a higher affinity for cumene hydroperoxide over the referred peroxides but cannot reduce linoleic acid hydroperoxide. Furthermore, *M. tuberculosis* Tpx reduces peroxynitrite (237). Phenotypical studies showed that the *tpx* mutant strains of *E. coli* (235, 238), *E. faecalis* (186), *S. typhimurium* (239) and *Streptococcus gordonii* (240) lose resistence to hydrogen peroxide. The *E. coli tpx* mutant was more sensitive to organic peroxides and paraquat, suffered higher levels of protein oxidation, and accumulated a higher concentration of lipid peroxides

when compared to the wild type (235, 238). Interestingly, the level of sensitivity to peroxides was higher for the *E. coli* and *S. typhimurium tpx* mutants relative to the *E. coli ahpC* and *bcp*, and *S. typhimurium ahpC* and *tsaA* mutants (239), suggesting a predominant role of this protein in oxidative stress protection. As referred above, Tpx is able to protect *H. pylori* against oxidative stress (section 3.2, (41, 46)) and in *C. jejuni*, both Tpx and Bcp proteins were shown important to combat oxidative and nitrosative stresses (183). The *M. tuberculosis* strains lacking *tpx* failed to grow and survive in activated macrophages (241). The *S. Typhimurium tpx* mutant has a lower intracellular proliferation in activated macrophages than the wild type strain (239). *E. faecalis* Tpx mediates a higher survival rate inside macrophages in comparison to AhpC (186). Altogether, Tpx have shown to be important *in vivo* for macrophage infection. The Tpx mutants of *H. pylori* (46), *M. tuberculosis* (241) and *E. faecalis* (186) were attenuated upon mice infection. Expression of *S. gordonii tpx* was up-regulated in the presence of oxygen and paraquat (240) and the *tpx* of *E. coli* was slightly induced by aeration (235). In *Mycobacterium bovis*, *tpx* is induced in response to thiol stress generated by exposure to diamide (242).

The Prx6 subfamily is present in archaea, bacteria and eukaryotes and their members are mainly 1-Cys peroxiredoxins, such as the *P. aeruginosa* LsfA (243) and the mammalian Prx6. Their principal structural trait is a C-terminal extension that is 15-40 residue longer than the characteristic C-terminal extension of Prx1/AhpC enzymes. All known Prx6 subfamily members are dimers and some form decamers (175). So far, the physiological role of bacterial Prx6 subfamily members was only studied for *P. aeruginosa* LsfA. The *P. aeruginosa lsfA* mutant strain is more sensitive than the wild type to hydrogen peroxide but not to organic peroxides. A redox sensitive fluorescent probe was more oxidized in the *lsfA* mutant-infected macrophages than in macrophages infected with the wild type strain, suggesting a role of LsfA in ROS protection. In an acute pneumonia model, mice infected with the *lsfA* mutant had increased cytokine release, neutrophil recruitment and increased survival compared to mice infected with the wild-type, which suggests that LsfA is able to downregulate the host innate immune response and contributes to pathogen colonization (243).

The Prx5 subfamily members occor in bacteria, fungi, plants and mammals. This subfamily of proteins that includes *Haemophilus influenzae* Prx5 (244) and the mammalian Prx5, usually form dimers independently of the redox state. Only approximately 17% of the known Prx5 subfamily members are 2-Cys and 20% of the proteins are fused with a glutaredoxin domain, which suggests that the glutaredoxin domain acts as the electron donor for the peroxiredoxin domain of these proteins (244). Interestingly, the human Prx5 exhibits peroxynitrite activity (245), but so far, the role of bacterial Prx5 members in oxidative and nitrosative stress protection was not studied.

The AhpE is the smallest peroxiredoxin subfamily, only present in *Mycobacteria* and other *Actinomycetes*. AhpE members are 1-Cys peroxiredoxins, form dimers, and have an extended loop at the N-terminus in relation to the other peroxiredoxin subfamilies (175). *M. tuberculosis* AhpE was reported to have peroxynitrite activity, reducing peroxynitrite by two orders of magnitude faster than hydrogen peroxide (246).

Table 3.4 summarizes the phylogenetic distribution, the main structural differences and oligomeric states of the different peroxiredoxin families.

Table 3.4 Phylogenetic distribution, oligomeric states and main structural differences among the different peroxiredoxin subfamilies

Subfamily	Phylogenetic distribution	Oligomeric states	Main structural differences	Location of C_R
Prx1/AhpC	Archaea, bacteria, plants, and other eukaryotes	Dimers/decamers/dodecamers	Extended C-terminus	C-terminus (99%)
Bcp/PrxQ	Archaea, bacteria, plants, and fungi (not animals)	Monomers/dimers	Extended helix α5	Helix α2 (61%) Helix α3 (6%)
Tpx	Bacteria	Dimers	N-terminal β hairpin	Helix α3 (>95%)
Prx6	Archaea, bacteria, plants, and other eukaryotes	Dimers/decamers	Long, extended C-terminus	No C_R (41%)
Prx5	Bacteria, plants, and other eukaryotes (not archaea)	Dimers	Fused with a glutaredoxin domain (~20%)	Helix α5 (21%) Between β1 and β2 of N-terminus (17%)
AhpE	Bacteria	Dimers	Extended loop at N-terminus	Helix α2 (67%). No C_R (19%)

Adapted from (175).

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Results

Chapter IV

FrxA is an S-nitrosoglutathione reductase enzyme that contributes to *Helicobacter pylori* pathogenicity

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Summary

Helicobacter pylori is a pathogen that infects the gastric mucosa of a large percentage of the human population worldwide, and predisposes to peptic ulceration and gastric cancer. Persistent colonization of humans by *H. pylori* triggers an inflammatory response that leads to the production of reactive nitrogen species. However, the mechanisms of *H. pylori* defence against nitrosative stress remain largely unknown. In this study, we show that the NADH-flavin oxidoreductase FrxA of *H. pylori*, besides metabolizing nitrofurans and metronidazole, has *S*-nitrosoglutathione reductase activity. In agreement with this, inactivation of the FrxA-encoding gene resulted in a strain that was more sensitive to *S*-nitrosoglutathione. FrxA was also shown to contribute to the proliferation of *H. pylori* in macrophages, which are key phagocytic cells of the mammalian innate immune system. Moreover, FrxA was shown to support the virulence of the pathogen upon mouse infection. Altogether, we provide evidence for a new function of FrxA that contributes to the successful chronic colonization ability that characterizes *H. pylori*.

4.1 Introduction

Helicobacter pylori is a human pathogen responsible for multiple gastric pathologies that, when untreated, may result in gastric cancer. The bacterium causes chronic infections of the gastric mucosa, owing to its ability to withstand exposure to harmful chemicals, such as the nitric oxide (NO) produced by the mammalian inducible NO synthase (iNOS) and derived from the nitrite present in the acidic stomach environment. Expression of iNOS is triggered by pathogen invasion of the phagocytic cells of the mammalian innate immune system, namely macrophages and neutrophils (1, 2). NO impairs the function of several bacterial metalloproteins by directly binding to metal centres, such as iron haem and iron-sulphur centres. Moreover, NO reacts rapidly with superoxide, produced by the mammalian NADPH oxidase, generating the potent oxidant HOONO (peroxynitrite), which also damages proteins and DNA. NO binds to thiol groups of cysteines of proteins to form S-nitrosothiols; this represents a form of reversible post-translational damage of cellular proteins (S-nitrosylation). Furthermore, toxic *S*-nitrosoglutathione (GSNO) is

generated inside cells by the univalent oxidation of NO to nitrosium ion, promoted by intracellular free or protein-bound metals, and further ligation to glutathione (3-5).

In several bacteria, the enzymatic removal of NO is performed by the flavodiiron NO reductases and flavohaemoglobins (6, 7), which are apparently absent in the genome of *H. pylori*. Recently, we reported that *H. pylori* uses a new type of NO reductase, NorH, that is present in all of the so far known *Helicobacter* species. These proteins constitute a new family of NO detoxifiers that occur in several microorganisms, including bacteria that contain the canonical enzymes (8).

Bacteria also alleviate the detrimental effects of nitrosative stress by reversing the S-nitrosylation process. In particular, the enzymatic reduction of GSNO to glutathione disulfide and ammonia, via a glutathione *N*-hydroxysulfenamide intermediate, is a reaction that allows control of the cellular S-nitrosothiol levels and protection from nitrosative stress (9). Endogenous GSNO reductase (GSNOR) (formally glutathione-dependent formaldehyde dehydrogenase) activity has been observed in *Escherichia coli* (10) and *Neisseria gonorrhoeae* (11). Also, glutathione-dependent class III alcohol dehydrogenases have been shown to catalyse GSNO reduction in *Neisseria meningitides* (11), *Hemophilus influenza* (12), and *Streptococcus pneumoniae* (13). Moreover, in *E. coli* (14) and *Mycobacterium tuberculosis* (15), the thioredoxin system, comprising thioredoxin, thioredoxin reductase, and NADPH, has emerged alongside GSNOR as a physiologically important denitrosylase. Likewise, both thioredoxins from *H. pylori* (thioredoxin 1 and thioredoxin 2) have been suggested to play a role in resistance to GSNO stress, owing to the increased susceptibility to GSNO of thioredoxin-deficient strains (16).

Treatment of *H. pylori* is achieved by double or triple antibiotic-based therapies that include the prodrug metronidazole. The drug belongs to the nitroimidazole family of antibiotics, and is activated by the microbial nitroreductases (17). *H. pylori* contains at least two nitroreductases, the oxygen-independent NADPH nitroreductase RdxA, and the NADH-flavin oxidoreductase FrxA (18-21).

Although there is general agreement that RdxA is involved in metronidazole activation, the correlation of metronidazole resistance with mutations in *frxA* is still

under debate, and the physiological role of the protein remains elusive. In this study, we tested whether *H. pylori* FrxA metabolizes other substrates, namely reactive nitrogen species. We produced recombinant FrxA, and characterized its enzymatic activity for reduction of nitrofurans, metronidazole, 3-nitrotyrosine, *S*-nitrocysteine, and GSNO. Furthermore, we generated a nonpolar *H. pylori frxA* mutant to determine the effect of *frxA* depletion on *H. pylori* survival *in vitro*, and in macrophages and mice. The results revealed new aspects of the function of FrxA as a GSNOR, and demonstrated the contribution of FrxA to macrophage infection and mouse colonization by *H. pylori*.

4.2 Results

FrxA reduces nitrofurans, metronidazole, and GSNO

To investigate the role of *H. pylori* FrxA, the protein was produced in *E. coli* cells and characterized. To this end, the *H. pylori frxA* gene was amplified by use of the oligonucleotides outlined in Table 1, and cloned into pET28a; the recombinant plasmid was then transformed into *E. coli* BL21-Gold (DE3). Cell growth and protein purification were performed as described in 'Experimental procedures'. Purified His-FrxA had an apparent molecular mass of ~ 26 kDa in SDS/PAGE (Figure 4.1a, inset), which agrees with the molecular mass derived from the gene sequence. The protein migrated as a dimer in gel filtration, with an estimated molecular mass of 52 kDa (Figure 4.1b).

Table 4.1 Oligonucleotides used in this study

Description	Oligonucleotide sequence (5'→3')
frxA gene deletion	
upstream flanking region	
frx1	CGCTCAAAAACCCCAAACATGC
frx2	TTATTCCTCCTAGTTAGTCATTTAATGTTCTCCTTTTTC
downstream flanking region	
frx3	TACCTGGAGGGAATAATGAGAATAAAAACGCTGTTAGC
frx4	TCTGAATACCTGAAAGGGCG
Real Time qRT-PCR	
ppk (*hp1010*)	
ppkRT5	GCGCGTTAGTCGTTTATGGCGTTT
ppkRT3	AGCGCTCAAAGGGTTGTAATTGCC
frxA (*hp0642*)	
frxART5	GGCTTGAACCATGGAAAATGC
frxART3	GGCTTGAACCATGGAAAATGC
Expression of FrxA	
FrxAndeI	CCTAAAGAATACTCGAGTGTGTTG
FrxAxhoI	GGAGAACATTCATATGGACAGAG

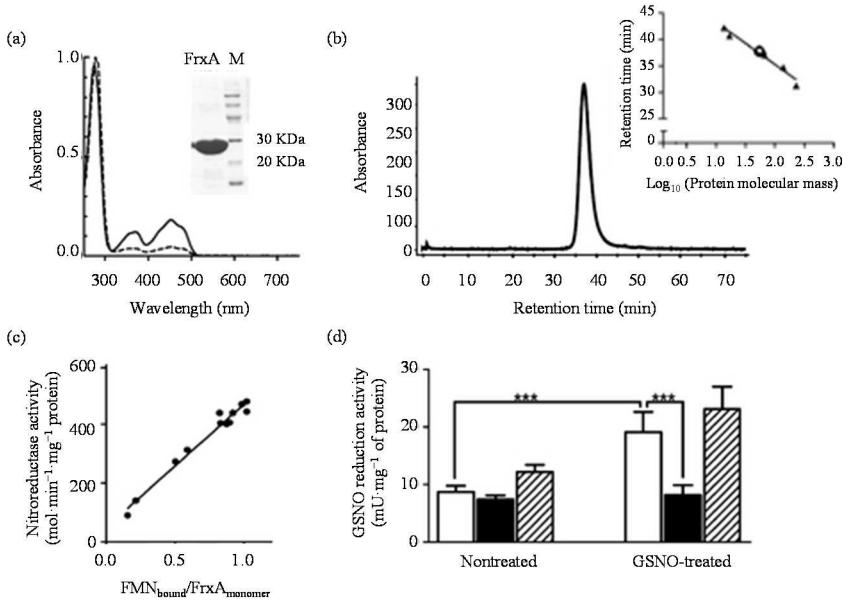

Figure 4.1 Biochemical characterization of *H. pylori* FrxA nitroreductase. (a) UV–visible spectra of *H. pylori* FrxA as isolated (dashed line) and after incubation with a

two-fold excess of FMN, i.e. containing one FMN per monomer (full line). Inset: SDS/PAGE with purified *H. pylori* FrxA (left lane) and a protein molecular mass marker (lane M). (b) Analytical gel filtration profile of *H. pylori* His-tagged FrxA. Inset: correlation between the molecular mass and retention time of protein standards (triangles); FrxA was eluted with an approximate molecular mass of 52 kDa (circle). (c) Dependence of the metronidazole-specific activity *H. pylori* FrxA (0.5 μM) on the FMN bound to the protein. The FMN content was determined as described in "Experimental procedures", and is depicted as the FMN bound/protein monomer ratio. Linear regression, R^2 = 0.97; and slope of 418.3 ± 22.2 nmol·min^{-1}·mg^{-1} protein. Activities were assayed with metronidazole (0.15 mM) and 0.2 mM NADPH, and are represented as nmol·min^{-1}·mg^{-1} protein. Two independent protein samples were assayed in triplicate. (d) Cells of wild type *H. pylori* 26695 (white bar) and the *frxA* (black bar) and *rdxA* (striped bar) mutants were left untreated and exposed for 1 h to 200 μM GSNO. GSNOR activity was determined in bacterial suspensions, prepared as described in 'Experimental procedures', with 200 μM NADPH and 400 μM GSNO. Values are means ± standard deviations of three biological cultures. Units represent 1 μmol of GSNO and NADPH consumed per minute. ***P < 0.001 (two-way ANOVA and Tukey's multiple comparison test).

The purified protein was yellow, and its UV-visible spectrum showed two bands in the 300-500-nm region, as is typical for flavin-containing proteins (Figure 4.1a). Treatment of FrxA with SDS induced the release of a cofactor, whose free form showed a visible spectrum with an absorbance maximum at 446 nm, which is characteristic of FMN molecules. The as-purified FrxA containing 0.2 FMN molecules per FrxA polypeptide chain, and incubation of the protein with an approximately two-fold concentration of FMN yielded a protein with one FMN per monomer.

The substrate specificity of FrxA for several nitro-substituted compounds, namely nitrofurantoin, nitrofurazone, metronidazole, and 3-nitro-L-tyrosine, was studied. FrxA showed NADPH-dependent activity for nitrofurans that varied from 190 to 1100 nmol·min^{-1}·mg^{-1} protein (Table 2), with the highest value being

observed for nitrofurazone; these activities are within the range of values found for other bacterial nitroreductases (22).

Table 4.2 Kinetic parameters of *H. pylori* FxrA enzyme activity for nitro-substituted and nitroso-substituted substrates

Substrate	V_{max} (nmol· min^{-1}· mg^{-1} protein)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Metronidazole	621 ± 38	22.4 ± 5.2	0.6	2.5 x 10^4
Nitrofurantoin	538 ± 67	42.3 ± 12.1	0.5	1.1 x 10^4
Nitrofurazone	1122 ± 83	14.3 ± 3.9	1.0	7.1 x 10^4
3-nitrotyrosine	190 ± 16	62.6 ± 13.7	0.1	2.7 x 10^3
GSNO	575 ± 24	4.1 ± 1.1	0.5	12.6 x 10^4
S-nitrosocysteine	716 ± 35	7.2 ± 2.0	0.6	8.9 x 10^4

FrxA had a metronidazole activity of 621 nmol· min^{-1}· mg^{-1} protein, which is comparable with the activities measured for nitrofuran compounds (Table 2). As the metronidazole activity of FrxA was lower than that reported for *H. pylori* RdxA (20), we tested whether the activity of FrxA was a function of the flavin content of the protein. It was observed that metronidazole activity of the isolated protein containing 0.2 FMN molecules per polypeptide chain accounted for ~ 20% of the activity of the full FMN-loaded FrxA, and that the catalytic activity increased with the FMN content of FrxA (Figure 4.1c).

Interestingly, FrxA was catalytically active towards 3-nitro-L-tyrosine (Table 2). This compound is a biologically relevant substrate that results from the damage induced in proteins by nitrosative stress and that is widely used as a marker of this type of stress.

Additionally, FrxA was able to reduce GSNO and S-nitrosylated cysteine with comparable kinetic parameters (Table 2). These activities were determined in the presence of NADPH, and reactions were initiated by the addition of the enzyme and monitored following the oxidation of NADPH and S-nitrosothiols. FrxA showed GSNOR activity of 575 nmol· min^{-1}· mg^{-1} protein; although this value is of the same order of magnitude as the values determined for other tested substrates, the lower K_m

and higher K_{cat}/K_m values for GSNO suggest that S-nitrosothiols may be the preferred substrates of *H. pylori* FrxA.

Altogether, the data revealed that, despite being a typical member of the nitroreductase family of homodimeric flavoproteins, FrxA shows significant GSNOR activity.

The *frxA* mutants are defective in GSNOR activity

As recombinant FrxA is a GSNOR, we evaluated its contribution to the GSNO reduction rate of *H. pylori* cells. To this end, the *H. pylori* 26695 strain carrying a deletion in *frxA* was constructed and its GSNOR activity was determined. For comparison purposes, an *H. pylori* line with inactivation of *rdxA* was also constructed and analysed. GSNOR activity was evaluated in mixtures containing cell extracts, which were prepared as described in 'Experimental procedures', buffer, and NADPH. The reaction was initiated by addition of GSNO. The GSNOR activities of *H. pylori* cells of wild type and mutant strains unexposed to stress showed no significant differences. However, whereas upon exposure to GSNO the *rdxA* mutant showed GSNOR activity comparable to that of the wild type, the *frxA* mutant showed lower GSNOR activity (Figure 4.1d).

The *frxA* mutants are hypersensitive to GSNO

Next, we tested whether FrxA contributes to the cellular GSNO resistance of *H. pylori* (Figure 4.2). Liquid cultures of *H. pylori* 26695 wild type and *frxA* mutant strains were inoculated and subjected to GSNO stress. In the absence of stress, the viability of the *frxA*-deficient strain was comparable to that of the parental strain, but for cells treated with GSNO the *frxA* mutant showed lower viability than the wild-type (Figure 4.2a). Interestingly, susceptibilities of the *frxA* mutant and parental strains were similar for cells exposed to the NO donor spermine-NONOate (data not shown).

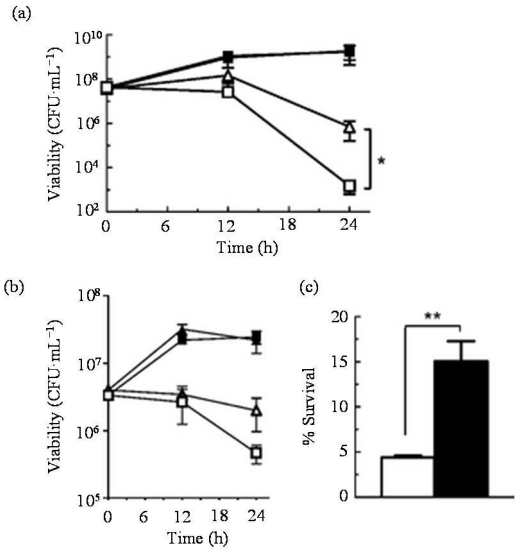

Figure 4.2 FrxA affords protection to *H. pylori* against nitrosative stress. (a) Cell viability of wild type *H. pylori* 26695 (triangles) and the *frxA* mutant (squares) cultivated in BHI medium supplemented with 10% fetal bovine serum in the absence (filled symbols) and in the presence (open symbols) of 200 μM GSNO. (b) Cell viability of wild type *H. pylori* B128 (triangles) and the *frxA* mutant (squares) cultivated in BHI medium supplemented with 10% fetal bovine serum in the absence (filled symbols) or presence (open symbols) of 300 μM GSNO. (c) Survival of the *H. pylori* B128 *frxA* mutant strain transformed with the empty vector pILL2157 (open bar) and with pILL2157 expressing *frxA* (filled bar), cultivated in BHI medium supplemented with 10% fetal bovine serum and 1 mM IPTG, after 24 h of exposure to 300 μM GSNO. Values are means ± standard deviations of three independent cultures. **P* < 0.05, ***P* < 0.01 (*t*-test).

To substantiate the results, the *frxA* gene deletion was introduced into another *H. pylori* strain, namely *H. pylori* B128, that has the ability to colonize mice (23). Identical growth assays were performed, and the results revealed that deletion of *frxA* impaired the resistance of *H. pylori* B128 to GSNO (Figure 4.2b). In addition, complementation experiments showed that expression of FrxA from the inducible

plasmid pILL2157-*frxA* was sufficient to rescue the phenotype of *H. pylori* B128 wild type (Figure 4.2c).

Interestingly, the *H. pylori* B128 *frxA* mutant proved to be more resistant to GSNO than *H. pylori* 26695, which may be attributable to a different genetic background, as high genetic variability is usually observed among *H. pylori* isolates.

Transcription of *frxA* is induced by nitrosative stress

The mRNA abundance of *frxA* was examined, with quantitative real-time RT-PCR, in cells of wild-type *H. pylori* treated with NO donors, namely GSNO and spermine-NONOate. The data showed that *frxA* expression underwent fold increases of 3.5 ± 0.6 and 5.0 ± 1.3 when cells were exposed to GSNO and spermine-NONOate, respectively.

The *frxA* mutant has reduced survival in macrophages

The decreased resistance of the *frxA* mutant to GSNO as compared with the parent strain led us to study the role of FrxA in the survival of *H. pylori* during interaction with macrophages (Figure 4.3a, b). A lower number of viable bacterial cells were recovered from macrophages infected with the *H. pylori* 26695 *frxA* mutant (Figure 4.3a) than from macrophages infected with the parental strain, which was assayed under the same experimental conditions.

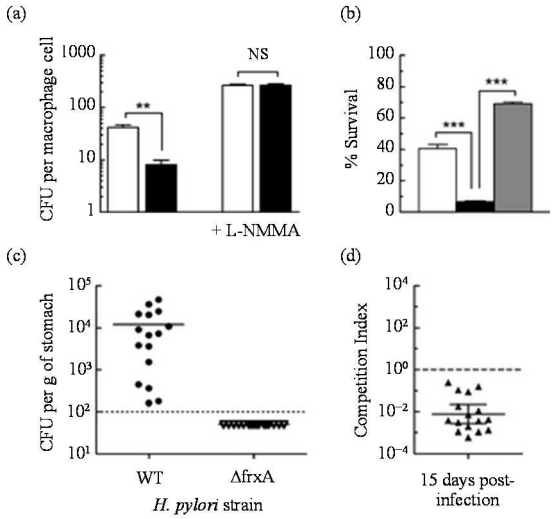

Figure 4.3 FrxA confers *in vivo* protection to *H. pylori*. (a) Survival of wild type *H. pylori* 26695 (open bar) and the *frxA* mutant (filled bar) after 24 h of infection of RAW264.7 macrophages (left) and after 24 h of infection of RAW264.7 macrophages treated with the iNOS inhibitor L-NMMA. Values are means ± standard errors (n = 9). $^{**}P < 0.01$ (one-way ANOVA). (b) Infection of RAW264.7 macrophages with wild type *H. pylori* B128 (white bar), the *frxA* mutant harbouring the empty vector pILL2157 (black bar), and the *frxA* mutant complemented with the vector pILL2157 expressing *frxA* (grey bar). Values are means and standard errors of the average values (n = 3). $^{***}P < 0.0001$ (ANOVA and Bonferroni test). In all assays, macrophages were seeded at 5×10^5 in 24-well plates and infected at a multiplicity of infection of 100. (c) Colonization of C57BL/6J mice with wild type *H. pylori* B128 (circles) and the *frxA* mutant (triangles). Mice were inoculated orally with a 1:1 mixture of wild-type *H. pylori* B128 and the *frxA* mutant (2×10^8 bacteria), and the stomach bacterial load was evaluated after 15 days of infection. Each data point corresponds to a single mouse (n = 16); the horizontal bar represents the mean, and the dashed line indicates the detection limit of CFUs per gram of stomach. (d) Competition index of the *H. pylori frxA* mutant strain upon coinfection of 15 mice. Each data point corresponds to a value calculated from the colonization level depicted in (c).

In order to correlate the lower resistance of the *frxA* mutant strain with an impaired capacity to withstand iNOS-dependent NO stress, experiments were repeated in the presence of an iNOS inhibitor. Macrophages incubated with NG-monomethyl-L-arginine (L-NMMA) that do not produce NO, were less efficient at eliminating *H. pylori*. Moreover, no differences were seen between the survival of the wild-type strain and that of the mutant strain (Figure 4.3a).

Infection assays performed with the *H. pylori* B128 *frxA*-deficient strain also showed that the mutant was killed more efficiently by macrophages to a degree comparable to that observed for the *H. pylori* 26695 *frxA* mutant (Figure 4.3b). Furthermore, complementation assays performed with the inducible plasmid pILL2157-*frxA* revealed that expression *in trans* of FrxA in the *H. pylori* B128 *frxA* mutant strain restored the survival of *H. pylori* in macrophages to levels resembling those observed for the wild type (Figure 4.3b).

These results demonstrate that FrxA deficiency impairs the proliferation of *H. pylori* in macrophages by promoting pathogen clearance.

FrxA protects *H. pylori* during mouse infection

To further evaluate the contribution of FrxA to successful infection by *H. pylori*, we determined the mouse colonization efficiency of the *frxA* mutant during competition with the wild type strain. C57BL/6J mice were infected orally with an inoculum consisting of a 1:1 mixture of viable cells of the *frxA* mutant and wild type strains. The level of gastric colonization was analysed in 16 mice at the peak of infection (15 days), which is characterized mainly by the infiltration of innate immune cells. At this time, mice were killed and the stomach homogenates were plated for viability determination. For each mouse, the competition index of the *frxA* mutant versus the wild type was calculated.

The results showed displacement of the mutant strain, which was almost completely eliminated after 15 days of infection (Figure 4.3c). Furthermore, the competition indexes determined for the 16 C57BL/6J mice were all below 1 (Figure 4.3d). These

data revealed that inactivation of *frxA* made *H. pylori* less proficient in colonizing the stomachs of mice.

4.3 Discussion

The level of the *H. pylori* FrxA nitroreductase activity determined in this study is similar to that reported previously (~ 0.5 μmol· min^{-1}· mg^{-1} protein), and is consistent with the observation that overexpression of *H. pylori* FrxA confers nitrofurantoin and nitrofurazone reductase activity to a nitroreductase-deleted *E. coli* strain (19). Here, we have also shown that *H. pylori* FrxA catalyses the reduction of 3-nitrotyrosine, a compound that is formed upon protein tyrosine nitration. To the best of our knowledge, such activity has so far only been tested for *E. coli* nitroreductases NsfA and NfsB, but neither was able to metabolize this substrate (24).

Despite the general agreement that the oxygen-independent NADPH nitroreductase RdxA and the NADH-flavin oxidoreductase FrxA of *H. pylori* are involved in metronidazole resistance, until now the metronidazole activity of FrxA could not be evaluated. In this study, we successfully measured this activity, and showed that the FMN-containing *H. pylori* FrxA has a metronidazole activity of 0.6 μmol· min^{-1}· mg^{-1} protein. It is possible that the lack of metronidazole activity previously reported for *H. pylori* FrxA (19) was attributable to the urea denaturation protocol utilized for purification, which may have led to the loss of the flavin cofactor required for the catalytic activity of FrxA.

H. pylori FrxA shows a metronidazole activity (0.6 μmol· min^{-1}· mg^{-1} protein) that is lower than that reported for the RdxA nitroreductase of *H. pylori* (9 μmol· min^{-1}· mg^{-1} protein) (19). In accordance with this observation, the *H. pylori frxA* mutant showed a lower level of metronidazole resistance than the *rdxA*-deficient strain (18).

It is noteworthy that our work also provides the first evidence that FrxA plays a role in protection of *H. pylori* against reactive nitrogen metabolites, as the protein has significant GSNOR activity (0.6 μmol· min^{-1}· mg^{-1} protein). In particular, FrxA shows a K_m for GSNO (4 μM) that is higher than that of the *E. coli*

formaldehyde-dependent GSNOR (700 μM) (10), indicating a higher affinity of FrxA for this substrate.

We previously reported that the *Staphylococcus aureus* NtrA nitroreductase has GSNOR activity (22); this dual activity of the two enzymes is consistent with several studies showing that nitroreductases may accept a wide range of substrates (25). However, it is interesting that neither protein shows activity towards NO gas, indicating specificity for GSNO.

Microbial nitroreductases are usually classified into classes A and B, typified by the *E. coli* enzymes NfsA and NfsB, respectively, and class C, which includes *S. aureus* NtrA (22). *H. pylori* FrxA has a high degree of amino acid sequence similarity with *E. coli* NfsB (18% identity; 41% similarity) and only 7% identity and 18% similarity with *S. aureus* NtrA. Hence, the bifunctional character of a nitroreductase and, in particular, its involvement in nitrosative stress defence cannot be inferred from the class in which the enzyme is included. Hence, it is possible that other bacterial nitroreductases may play an equivalent role in nitrosative detoxification.

Transcription of *frxA* is increased in cells exposed to GSNO and spermine-NONOate, which are compounds commonly utilized as NO donors. Induction of transcription of the FrxA-encoding gene by nitrosative stress is compatible with the reduced viability and GSNO reduction capability of *H. pylori frxA* mutant cells. This result is in agreement with the general observation that S-nitrosothiols elicit a transcriptional response of gene products involved in nitrosative protective functions, even of gene products that do not detoxify NO directly (26-28).

Although *H. pylori* lacks the machinery to synthesize glutathione, it was reported to utilize the host's glutathione pool and to contain free cysteine (29, 30). Hence, the capacity of FrxA to reduce both GSNO and *S*-nitrosocysteine may represent an advantageous adaptation for survival in environments that contain the two compounds.

Our results show that the growth of *H. pylori* is significantly inhibited by treatment with 200 μM GSNO, *i.e.* even at concentrations lower than those tested in

previous studies (16). S-nitrosylation of a number of proteins of *H. pylori* has been implicated in the susceptibility of the bacterium to NO. These include alkyl hydroperoxide reductase and the urease α-subunit, and the latter was shown to be reversibly inhibited by GSNO in a concentration-dependent manner (31). Owing to its GSNOR activity, FrxA is expected to contribute to the protection of urease and to the *in vivo* survival of *H. pylori*. Indeed, deletion of *frxA* is herein shown to decrease the resistance of the bacterium during macrophage infection and to compromise the pathogen´s ability to sustain mouse stomach colonization. Although this was not an expected result, considering the canonical function of nitroreductases as activators of metronidazole, the demonstrated GSNOR activity of FrxA allows clarification of its association with a susceptible phenotype upon mouse colonization (32). The data also explain the rare occurrence of FrxA mutations in metronidazole-resistant clinical strains, as inactivation of its activity is expected to be detrimental to *H. pylori* survival. Therefore, it may be speculated that targeting FrxA with drugs or antibodies could constitute an adjunct to the existing antibiotics by facilitating host-dependent clearance of the pathogen.

In summary, the findings of this study have revealed a novel mechanism for the metabolism of S-nitrosothiols by *H. pylori*, a poorly understood aspect of this pathogen. We demonstrate that, despite the fact that FrxA seems to be similar to other nitroreductases in relation to nitrofuran reduction, it is distinct in its ability to reduce GSNO. We have proved that FrxA contributes to the survival of *H. pylori* during macrophage infection and mouse gastric colonization. Hence, FrxA constitutes an *H. pylori* defence against the S-nitrosothiol stresses imposed by the host mammalian immunity to which the pathogen is exposed during its lifelong colonization.

4.4 Experimental procedures

Reagents, bacteria, culture conditions, and plasmids

All reagents were acquired from Sigma, unless otherwise stated. *H. pylori* 26695 and B128 were used as wild type strains, and were routinely cultivated at 37 °C in a microaerobic atmosphere (6% O_2, 7% CO_2, 3.5% H_2, and 83.5% N_2) (Anoxomat Mart, Drachten, The Netherlands), in horse blood agar (BA) composed of Blood Agar

Base no. 2 (Oxoid, Basingstoke, UK) with 10% (v/v) defibrinated horse blood (Probiológica, Lisbon, Portugal), or brain heart infusion (BHI) broth (Oxoid) supplemented with 10% (v/v) decomplemented fetal bovine serum (Gibco-Invitrogen, Paisley, Scotland, UK). All media were supplemented with an antibiotic/antifungal mix composed of 0.3 $mg \cdot L^{-1}$ polymyxin B, 12.5 $mg \cdot L^{-1}$ vancomycin, 6 $mg \cdot L^{-1}$ trimethoprim and 5 $mg \cdot L^{-1}$ amphotericin B, and, when required, 20 $\mu g \cdot mL^{-1}$ kanamycin and 4 $\mu g \cdot mL^{-1}$ chloramphenicol. *H. pylori* grown for 24 h in BA plates was utilized to inoculate liquid precultures (25-cm^2 cell culture flasks filled with 10 mL of BHI + fetal bovine serum medium) at a OD_{600} of 0.05 that were grown for 16 h at 37 °C and 150 r.p.m. (final OD_{600} of ~ 1). The resultant cells served as inocula in all subsequent studies.

Plasmid manipulations were performed in *E. coli* XL2-Blue and *E. coli* MC4100. The triparental conjugation was performed with *E. coli* GC7 (pBRK2013), and *E. coli* BL21 Gold (DE3) served as host cells for expression of the recombinant protein. All *E. coli* cells were grown on LB medium that, when required, was supplemented with 30 $\mu g \cdot mL^{-1}$ kanamycin, 10 $\mu g \cdot mL^{-1}$ tetracycline, and 30 $\mu g \cdot mL^{-1}$ chloramphenicol.

GSNO and *S*-nitrosocysteine were freshly prepared by mixing equimolar amounts of sodium nitrite and reduced glutathione and cysteine, respectively, under acidic conditions (0.05 M HCl), and neutralized with NaOH (33).

Liquid precultures of *H. pylori* 26695 and B128 wild type strains and their corresponding *frxA* (*hp0642* in *H. pylori* 26695) mutants were transformed with the empty plasmid pILL2157 and pILL2157 containing the *frxA* gene. Assays were performed in 24-well plates containing 1.2 mL of BHI + fetal bovine serum medium and, when indicated, supplemented with isopropyl thio-β-D-galactoside (IPTG). Plates were inoculated with the precultures to an initial OD_{600} of 0.05, and immediately exposed to 200 μM and 300 μM GSNO or left untreated (control). The number of viable cells [colony-forming units (CFUs)$\cdot mL^{-1}$] was monitored after 12 h and 24 h by serial dilution of the cultures in BHI medium and plating on BA plates. The percentage survival represents the ratio of the number of viable cells determined in treated and untreated cells.

Construction of the *H. pylori* nonpolar *frxA*-deficient mutant

Nonpolar inactivation of *frxA* and *rdxA* in *H. pylori* 26695 and B128 was performed by allelic exchange after transformation of *H. pylori* with a three-fragment assembly product that carried *frxA* flanking regions of the target gene and the kanamycin *aphA*-3 cassette, with the primers shown in Table 1. Generation of the product and its introduction into *H. pylori* were performed essentially as previously described (8). Inactivation of *rdxA* was achieved by transforming *H. pylori* with a previously constructed suicide plasmid that carries the kanamycin cassette flanked by 500-bp regions upstream and downstream of *rdxA* (34). The presence of the desired mutations in the *H. pylori* chromosomal DNA was confirmed by PCR analysis.

For the complementation assays, *frxA* was amplified from the *H. pylori* 26695 genomic DNA with primers FrxAndeI and FrxAxhoI (Table 1), digested with *Nde*I/*Xho*I, and cloned into *Nde*I/*Xho*I-digested pILL2157 (35) to give recombinant pILL2157-*frxA*. pILL2157 and pILL2157-*frxA* were introduced by triparental conjugation into *H. pylori* B128 *frxA* with *E. coli* GC7 (pRK2013) as mobilizer (36).

Production and purification of recombinant FrxA

The *frxA* gene was PCR-amplified from the *H. pylori* 26695 genomic DNA with primers FrxAndeI and FrxAxhoI (Table 1), and cloned into *Nde*I/*Xho*I pET28a (Novagen, Nottingham, UK) to produce a protein with a poly-histidine tail fusion at the N-terminal region. The resulting vector, pET28-*frxA*, was introduced into *E. coli* BL21 Gold (DE3), which was grown aerobically at 37 °C in LB medium supplemented with 50 μM riboflavin. When they had reached a OD_{600} of 0.3, cells were treated with 500 μM IPTG and grown for a further 6 h at 20 °C. Cells were then harvested by centrifugation (8000 ***g***, 10 min at 4 °C), disrupted in a French pressure cell, and ultracentrifuged for 2 h at 100,000 ***g*** and 4 °C. The soluble fraction was loaded into a Ni^{2+} -chelating Sepharose Fast Flow resin (GE Healthcare, Little Chalfont, UK) previously equilibrated with 20 mM Tris/HCl (pH 7.5), 500 mM NaCl, and 10% (v/v) glycerol, and FrxA was eluted with 500 mM imidazole. After

overnight dialysis against 20 mM Tris/HCl (pH 7.5) containing 10% (v/v) glycerol, the protein purity was evaluated by SDS/PAGE with the Roti-Mark protein molecular mass marker (Carl Roth). The protein concentration was determined with the bicinchoninic acid assay (37).

The quaternary structure of FrxA was determined in a Tricorn Superdex 200 (10/300) GL column (GE Healthcare), equilibrated in 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, and 10% (v/v) glycerol, with catalase (230,000 Da), myoglobin (17,000 Da), haemoglobin (64,000 Da), γ-globulin (140,000 Da), horse cytochrome *c* (13,700 Da) and albumin (66,000 Da) as standards.

Incorporation of FMN was achieved by incubating the purified *H. pylori* FrxA (20 μM) with 10-40 μM FMN for 20 min at room temperature under light-protective conditions. Unbound FMN was removed by passing the sample through a Biospin P-6 column (Biorad, Munich, Germany). The type and content of flavin was determined after incubation of FrxA (~ 20 μM) with SDS 0.2% (w/v) for 20 min, by measuring the absorbance at 446 nm [$\varepsilon_{446\,nm}$ (FMN) = 12,000 $M^{-1}\cdot cm^{-1}$] (38).

Enzymatic assays

H. pylori cultures grown for 24 h on BA plates were resuspended in BHI medium supplemented with 0.2% (v/v) β-cyclodextrin to a OD_{600} of ~ 0.4, and left untreated or exposed to 100 μM GSNO for 1 h at 37 °C. Cells were then collected, washed, and resuspended in reaction buffer, and lysates were prepared by incubating cells with 0.1 $mg\cdot mL^{-1}$ lysozyme and 0.02% sodium deoxycholate for 15 min at 37 °C. Reaction mixtures containing the cell lysate, 20 mM Tris/HCl (pH 7.5), 0.2 mM NADPH and 0.4 mM GSNO were used to determine GSNOR activity by monitoring the combined consumption of NADPH and GSNO at 340 nm [$\varepsilon_{340\,nm}$ (NADPH + GSNO) = 7.04 $mM^{-1}\cdot cm^{-1}$) (8). In all cases, NADPH consumption of reaction mixtures lacking the cell lysate was evaluated and subtracted from that obtained for the respective reaction mixtures containing all components. Activities are defined as units (μmol GSNO consumption$\cdot min^{-1}\cdot$ mg total $protein^{-1}$). At least three biological samples were always analysed.

The enzymatic activity of purified *H. pylori* FrxA (0.5 μM) was determined for the indicated substrates (2.5-200 μM) and evaluated in reaction mixtures that contained 50 mM Tris/HCl buffer (pH 7.5) and 200 μM NADPH; the reaction was initiated by the addition of FrxA, which was previously incubated with a two-fold molar excess of FMN. Metronidazole activity was determined with FrxA previously incubated with the indicated amounts of FMN, as described above. Values were corrected for the activity of the reaction between NADPH and the corresponding substrate.

The following substrates were assayed: nitrofurazone, nitrofurantoin, metronidazole, and 3-nitrotyrosine. GSNO and *S*-nitrosocysteine, freshly prepared from reduced glutathione or cysteine, as described above, were also tested as substrates. All assays were performed in 96-well plates (200 μL per well) in a Multiskan Go microplate spectrophotometer (Thermo Scientific, Dreieich, Germany). The following extinction coefficients were used: $\varepsilon_{(\text{nitrofurazone, 400 nm})} = 12.96$ $mM^{-1} \cdot cm^{-1}$ (39); $\varepsilon_{(\text{nitrofurantoin, 420 nm})} = 12.0$ $mM^{-1} \cdot cm^{-1}$ (20), $\varepsilon_{(\text{3-nitrotyrosine, 420 nm})} = 4.40$ $mM^{-1} \cdot cm^{-1}$ (40), $\varepsilon_{(\text{NADPH, 340 nm})} = 6.22$ $mM^{-1} \cdot cm^{-1}$, $\varepsilon_{(\text{metronidazole, 320 nm})} = 9.0$ $mM^{-1} \cdot cm^{-1}$ (20), and $\varepsilon_{(\text{GSNO/CysNO, 334 nm})} = 0.907$ $mM^{-1} \cdot cm^{-1}$ (33). Two independent batches of protein were assayed in triplicate, and data were fitted to the Michaelis-Menten equation with GraphPad Prism 5.

Quantitative real-time RT-PCR analysis

An overnight culture of *H. pylori* 26695 was utilized to inoculate fresh BHI + fetal bovine serum medium to a OD_{600} of 0.05. Cells were grown to a OD_{600} of 0.5, and cultured for 1 h in the absence and in the presence of 150 μM spermine-NONOate or 200 μM GSNO. After collection of the cells by centrifugation (8000 ***g***, 10 min at 4 °C), the total RNA was isolated with the Qiagen RNeasy kit (Qiagen, Hilden, Germany), and residual DNA was removed by treatment with Turbo DNA-free (Ambion, Madrid, Spain). RNA was quantified in a NanoDrop spectrophotometer, and its integrity was confirmed by agarose gel electrophoresis. cDNA was prepared by the use of total RNA (2 μg), random hexamers (100 ng; Roche, Mannheim, Germany), and Superscript II Reverse Transcriptase (Invitrogen, Paisley, Scotland,

UK). The quantitative real-time RT-PCR reactions were prepared with the LightCycler FastStart DNA Master SYBER Green I kit, with 20 ng of cDNA and oligonucleotides frxART5 and frxART3 (Table 1), and performed in a LightCycler instrument (Roche Applied Science). The *frxA* transcript level was normalized to the mRNA abundance of the constitutive *ppk* gene (polyphosphate kinase; *hp1010*) determined with the oligonucleotides ppkRT5 and ppkRT3 (Table 1). Three biological samples were analysed in duplicate.

Macrophage assays

Murine macrophage RAW264.7 cells (ATCC Tib71) were cultured in DMEM with 4.5 $g \cdot L^{-1}$ glucose and glutamax supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, 70 $units \cdot mL^{-1}$ penicillin and 70 $\mu g \cdot mL^{-1}$ streptomycin in a humidified 5% CO_2/95% air atmosphere. Macrophages were seeded on 24-well plates (5×10^5 cells per well), and after 24 h the medium was replaced with infection medium, namely DMEM (Gibco) supplemented with 1 mM sodium pyruvate and 0.4 mM L-arginine, and, when required, 0.8 mM L-NMMA. Wild type and respective *frxA* mutants of *H. pylori* 26695 and B128 previously transformed with pILL2157 and pILL2157-*frxA* were grown for 24 h in BA plates and used to prepare bacterial suspensions, which were added to the macrophages at a multiplicity of infection of 100. The plates were then centrifuged at 1500 ***g*** for 5 min, and incubated for 24 h in the presence of 1 mM IPTG. For bacterial survival determination, cells were scraped from wells, diluted in BHI medium, and added to BA plates, which were next incubated under microaerobic conditions at 37 °C. Aliquots of the supernatants were taken to evaluate the nitrite concentration by means of the Griess reaction method (41); the standard sodium nitrite solution was prepared in infection medium.

Mouse colonization assays

Animal handling and experiments were carried out in strict accordance with institutional guidelines prescribed by the committee of Hygiène Sécurité et Protection de l'Environnement (Protocol 98-223; Institut Pasteur) and the European Union Directive 2010/63/EU (and its revision 86/609/EEC) on the protection of animals

used for scientific purposes. Our laboratory has the administrative authorization for animal experimentation (Permit Number 75-1554). All efforts were made to minimize suffering. Mice were killed with CO_2.

For mouse infection, bacterial suspensions of *H. pylori* B128 and the derived *frxA* mutant were independently prepared in physiological saline solution with, as inocula, cultures of *H. pylori* cultivated in BA plates for 24 h. Infection was performed orally by feeding 5-week-old female wild-type C57BL/6J mice with a bacterial suspension consisting of a mixture (1:1) of the *H. pylori* wild type and mutant strains (2×10^8 bacteria per mouse).

The numbers of viable cells of wild-type and *frxA* mutant *H. pylori* in the inoculation mixtures were assayed by plating serial dilutions of the cultures in kanamycin-selective and nonselective plates. After 15 days of infection, mice (n = 16) were killed with CO_2, and their stomachs were weighed before being ground in peptone broth and serially diluted in peptone broth. The viability of each *H. pylori* strain was evaluated from the CFUs$\cdot$mL^{-1} obtained upon plating of the stomach homogenates on BA medium containing 200 μg$\cdot$mL^{-1}bacitracin and 10 μg$\cdot$mL^{-1} nalidixic acid. Selection of the *frxA* mutant was achieved by further supplementation of the medium with 20 μg$\cdot$mL^{-1}kanamycin. Plates were incubated for 8 days at 37 °C in a microaerobic atmosphere. The competition index of *frxA* was calculated from the following formula:

$$\text{competition index} = \left(\mathrm{CFU}/\mathrm{CFU_{wt}}\right)_{\text{each mouse}} \Big/ \left(\mathrm{CFU}_{frxA}/\mathrm{CFU_{wt}}\right)_{\text{inoculum}}$$

Statistical analyses were performed with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) and one-way ANOVA, followed by a Bonferroni multiple comparison test; for the *t*-test, a significance threshold at $P < 0.05$ (95% confidence level) was used.

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Chapter V

Helicobacter pullorum induces in murine macrophages nitric oxide release that promotes phagocytosis and killing

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Adelina Margarida Parente performed all the experimental work with the exception of the semi-quantitative Real time PCR experiments. Adelina Margarida Parente also participated in the writing of the manuscript.

Summary

Helicobacter pullorum is an avian enterohepatic species that, more recently, has also been found as a naturally acquired infection in mice and rats, and isolated from patients with gastrointestinal and hepatobiliary diseases. In this work, the interaction between *H. pullorum* and murine macrophages was examined. Firstly, the impact of nitric oxide, which is an antimicrobial produced by mammalian macrophages, on *H. pullorum* 6350-92 viability and morphology was studied by colony-forming assays and light microscopy, respectively. Exposure to nitric oxide lowered *H. pullorum* viability, in a growth-phase-dependent manner, and decreased the mean cell size. However, the number of coccoid forms remained low, contrasting with what has been observed for other *Helicobacter* species. Confocal microscopy showed that *H. pullorum* is internalized by murine macrophages, triggering nitric oxide production that promotes phagocytosis and killing of the pathogen. Interaction between *H. pullorum* and macrophages stimulated secretion of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and MIP-2. These results show that *H. pullorum* is able to infect mammalian murine cells triggering an inflammatory response.

5.1 Introduction

Helicobacter pullorum is an enterohepatic Gram-negative and microaerophilic non-spore-forming bacterium that presents slightly curved rod cells and an unsheathed monopolar flagellum. *H. pullorum* was first described in the caeca of asymptomatic poultry and the livers and intestinal contents of laying hens suspected of having vibrionic hepatitis (1). *H. pullorum* was also shown to colonize turkeys, guinea fowls, psittacine birds and, more recently, rats and mice (2-8). Moreover, *H. pullorum* is considered an emergent human foodborne pathogen implicated in several intestinal pathologies (9). In particular, *H. pullorum* has been found in patients with diarrhoea, gastroenteritis, inflammatory bowel disease, hepatobiliary disease and hepatic cancer (10-16).

H. pullorum adheres to and invades human intestinal Caco-2 cells, possesses a type-VI secretion system (17) and expresses a cytolethal distending toxin (CDT) (18-20). The bacterium presents LPS with the highest biological activity within the genus *Helicobacter*, capable of inducing NF-*κβ* activation (21). In gastric and intestinal epithelial cell lines, *H. pullorum* triggers the production of IL-8 via NF-*κβ*, probably due to its LPS involvement (22).

As sentinel cells of the human body, macrophages are activated by several signals, including cytokines (e.g. gamma interferon, INF-γ) and microbial compounds (*e.g.* LPS). In primed macrophages, a variety of pro-inflammatory cytokines, such as IL-1β and TNF-α mediate the expression of the inducible nitric oxide synthase (iNOS) (23).

The mammalian iNOS enzyme produces nitric oxide (NO), a highly reactive molecule that combines with oxygen species, produced by activated macrophages and neutrophils, to generate species like nitrosonium (NO^+), nitroxyl (NO^-), peroxynitrite ($ONOO^-$) and nitrite (NO_2^-). Most of these reactive nitrogen species damage several bacterial components, such as DNA, lipids, protein metal centres and the amino acid residues of proteins, causing inactivation of key metabolic functions and ultimately leading to microbial clearance (24-26).

In spite of the potential pathogenicity of this bacterium, the mechanisms of *H. pullorum* infection remain poorly understood. For example, there is still a gap in the knowledge on how *H. pullorum* behaves when facing antimicrobials produced by the host immune system and regarding the response of phagocytes to *H. pullorum*. In this work, we have evaluated the viability and morphological modifications of *H. pullorum* when exposed to nitrosative stress generated by different NO donors applied at different stages of bacterial growth. The interaction of *H. pullorum* with murine macrophages was studied by confocal microscopy and through the analysis of the cytokines stimulated upon *H. pullorum* infection.

5.2 Experimental procedures

Bacterial strain manipulation

H. pullorum 6350-92 (CCUG 33838), which was isolated from a stool sample of a patient with gastroenteritis and hepatitis (27), was used as the reference strain. The strain was routinely cultivated on blood agar (BA) plates, which are composed of solid medium blood agar base no. 2 (Oxoid) supplemented with 10% (v/v) defibrinated horse blood (Probiológica) and an antibiotic/antifungal mix containing 6.3 g vancomycin L^{-1} (Roth), 3.1 g trimethoprim L^{-1} (Sigma) and 2.5 g amphotericin B L^{-1} (Roth). Cells were incubated in closed jars, at 37 °C, under a microaerobic atmosphere (7% CO_2, 6 % O_2, 3.5 % H_2 and 83.5 % N_2) generated by an Anoxomat system (Mart Microbiology). Bacteria were taken as fully grown after culturing on BA plates for a total of 5 days, during which bacteria were twice transferred to fresh BA medium.

Viability tests

The viability of *H. pullorum* under nitrosative stress conditions was determined by exposing cells to the following nitrosative stress generators: spermine-NONOate (Sigma; $t_{1/2}$ ~ 40 min; (28)), dipropylenetriamine (DPTA)-NONOate (Cayman; $t_{½}$ ~ 3 h) and *S*-nitrosoglutathione (GSNO; $t_{1/2}$ ~ 50 min; (29)). GSNO was freshly prepared by mixing equimolar amounts of sodium nitrite and reduced glutathione under acidic conditions (0.05 M HCl) (28). For these assays, fully grown bacteria were inoculated in 25 cm^2 cell culture flasks (Nunc) filled with 10 mL medium, at an initial optical density at 600 nm (OD_{600}) of 0.1-0.2, in brain heart infusion (BHI) broth (Oxoid) plus 10% (v/v) defibrinated FCS (Gibco-Invitrogen) (BHI-FCS) and grown for 19 h at 150 r.p.m. These cells were used to inoculate BHI medium supplemented with 0.2% (w/v) β-cyclodextrin (Sigma) (BHI-βCD) at an OD_{600} of 0.05, and distributed into 24-well plates. The stress generators were added, at concentrations ranging from 50 to 500 μM, at different stages of *H. pullorum* growth: immediately after inoculation (OD_{600} ~ 0.05, lag phase), after 12 h (OD_{600} ~ 0.2, early exponential phase) and after 48 h (OD_{600} ~ 1, stationary phase). The

number of viable cells was monitored at 12 h intervals up to 48 h after the stress application. The number of CFU·mL^{-1} was evaluated by serially diluting each sample in BHI medium and plating on antibiotic/antifungal cocktail containing BA plates, which were incubated for 48 h.

Morphological studies

H. pullorum morphology under nitrosative stress was studied in cells grown in BHI-FCS, as described above for the viability tests, and exposed to spermine-NONOate (10, 50 and 100 μM). Cells prepared similarly but not exposed to any stress were also visualized. After 24 h incubation, cell suspensions were pelleted by centrifugation (5 min at 2400 ***g***), resuspended in 10-50 μl PBS and mounted onto 1.7% (w/v) agarose-coated glass slides. Images were acquired with a iXonEMCCD+885 cooled camera (Andor Technology) attached to a DMR600 Microscope (Leica), with × 64/1.6 NA magnification, and treated with ImageJ software (30). The percentage of bacilli versus cocci forms and the cell size were evaluated by counting and measuring manually a total of 100 cells per condition using Metamorph software version 4 (Molecular Devices).

Infection assays with murine macrophages

Murine macrophages J774A.1 (ATCC TIB-67) were cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose, GlutaMAX supplement, pyruvate (GIBCO), 10% (v/v) FCS and 100 μg penicillin/streptomycin mL^{-1} (Sigma) (DMEMc medium), and incubated in a humidified 5% CO_2-air-controlled atmosphere at 37 °C. For the infection assays, J774A.1 macrophages were resuspended in Roswell Park Memorial Institute 1640 medium (RPMI), GlutaMAX supplement (Gibco) containing 10% (v/v) FCS and 100 μg penicillin/streptomycin mL^{-1} (RPMIc medium), and seeded for 3 h in 24-well plates at a density of 5×10^5cells per well. Macrophages were left untreated (non-activated macrophages), or activated overnight with 0.3 μg INF-γ mL^{-1}(Sigma) and 1 μg LPS mL^{-1} (Sigma) (activated macrophages). When required, murine iNOS activity was inhibited by NG-monomethyl-L-arginine acetate salt (L-NMMA; Sigma; 800 μM), which was added

simultaneously to INF-γ and LPS. After 12 h of incubation, the macrophage medium was exchanged by infection medium that consisted of RPMI GlutaMAX plus 10% FCS (RPMIi) and, where indicated, supplemented with 800 μM L-NMMA.

For macrophage infection assays, fully grown bacteria were cultured on BA plates for another 24 h. These bacteria were used to inoculate, at an OD_{600} ~ 0.1-0.2, Brucella broth liquid medium (BB; Gibco) containing 5% FCS (BB-FCS), and the *H. pullorum* was grown for 15 h. Bacilli were then pelleted (10 min, 8740 ***g*** , 4 °C) and resuspended in RPMIi at an OD_{600} of 0.2, and the bacterial viability determined by CFU counting prior to incubation within macrophages (time 0 of infection). Bacteria were used to infect macrophages, at a MOI of 100, for 5 and 10 h. For each time point, the *H. pullorum*-infected macrophages were collected and bacteria present in the supernatant, adherent to the surface of the macrophages and localized inside the macrophages were platted together, and the CFU count of viable *H. pullorum* bacilli was determined following incubation under microaerobic conditions. At the same time points of infection, the amount of NO produced by macrophages as the nitrite accumulated in the supernatants of the macrophage cell cultures was quantified. Nitrite production by macrophages that were not infected or infected with *H. pullorum*, and in the presence and in the absence of INF-γ/LPS/L-NMMA, was also analysed. The nitrite content was determined by microtitre plate colorimetric assay (Multiskan GO; Thermo Scientific), which consisted of readings of the absorbance at 540 nm of the 1:1 mixtures of supernatant (100 μL) and Griess reagent (1%, (w/v), sulfanilamide/0.1% (w/v) naphthylethylene diamine dihydrochloride/2% (v/v), phosphoric acid). Sodium nitrite was used as standard.

Confocal experiments

For the confocal experiments, J774A.1 macrophages (1×10^6 cells per well) were seeded in 6-well plates containing submerged glass coverslips and cultivated in DMEMc for 12 h. Macrophages were left untreated (non-activated macrophages) or activated for 5 h with 0.15 μg INF-γ mL^{-1} and 0.5 μg LPS ml^{-1} (activated macrophages); where indicated, the inhibition of the NO production was achieved by the addition of 800 μM L-NMMA simultaneously with INF-γ/LPS.

H. pullorum bacilli grown in BB-FCS for 15 h, as described above for the macrophage assays, were washed with PBS, resuspended in DEMEM without antibiotics (DMEMi) and DMEMi plus 800 μM L-NMMA at an OD_{600} of ~ 0.2 and viability was determined by CFU counting, and the cells were then used to infect macrophages at a MOI of 100. After 30 min and 2 h of infection, each well was washed three times with PBS to remove non-adherent bacteria, the cells were fixed with 4% (w/v) formaldehyde, washed again (three times in PBS), stained with 2 μM HCS CellMask red (Molecular Probes) for 30 min at room temperature and PBS washed three times. The coverslips were mounted onto microscopy slides and confocal *Z*-stacks were acquired on a Leica SP5 confocal microscope, using a × 63/1.3 NA oil immersion objective, a 568 nm laser line and the spectral detection adjusted for the emission of the Alexa 568 fluorochrome. Stained macrophages that were activated by INF-γ and LPS, and incubated in DMEMi for 2 h, were also examined, and served as internal controls. For quantitative assessment, adherent and intracellular bacteria of 100 random macrophages per condition were counted and images treated with ImageJ software. Two separate experiments were performed and cell counting was validated by two independent observers.

In parallel experiments, the viability of the adherent and intracellular *H. pullorum* bacilli was determined upon infection of macrophages for 30 min and 2 h. Each well was washed three times with PBS to remove bacteria present in supernatants, macrophages lysed with 2% (w/v) saponin and their bacterial content evaluated by CFU counting.

Analysis of cytokine gene expression

Expression of TNF-α, IL-1β, IL-6 and macrophage inflammatory protein 2 (MIP-2), which is the mouse homologue of the mammalian IL-8 (31), was quantified in mRNA extracted from J774A.1 macrophages seeded, for 3 h, in 24-well plates (5×10^5 cells per well) containing RPMIc medium. *H. pullorum* bacilli prepared in RPMIi, as described above for the macrophage assays, were incubated with macrophages at a MOI of 100, for 6 h. For comparison purposes, expression of cytokines in macrophages non-infected, non-activated and activated only with INF-

γ (0.15 μg·mL^{-1}) and LPS (0.5 μg·mL^{-1}) was also measured. After incubation, the wells were washed with cold PBS and the total RNA was isolated with a High Pure RNA isolation kit (Roche). Residual DNA was digested using the enzymes of the Turbo DNA-free kit (Ambion). For each sample, 200 ng RNA was converted to cDNA using the anchored-oligo-dT primers and the Transcriptor High Fidelity cDNA synthesis kit (Roche). Semiquantitative reverse transcriptase PCR reactions contained 40 ng cDNA μL^{-1}, 2.5 U *Taq* DNA polymerase (BioLabs), 200 μM dNTPs (Nzytech) and 0.5 μM of each primer (Table 5.1), in 50 μL final volume. The reverse transcriptase PCR conditions were: 1 cycle of denaturation at 95 °C (30 s-2 min), followed by 30 cycles at 95 °C for 45 s, 50-55 °C for 30-45 s and 68 °C for 1 min, followed by a final cycle at 68 °C for 5 min. Bio-Rad Quantity One software was used in order to perform three steps that allowed the determination of the relative quantification of the cytokine expression: (i) image background subtraction; (ii) estimation and integration of all the pixels present in the DNA bands to an estimated area; and (iii) the area obtained for the gene band of interest, under the different conditions tested, was divided by the area estimated for the constitutive gene band, the murine glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) gene, in the respective condition. These estimated relative quantifications enabled the establishment of comparisons of differences in cytokine expression upon macrophage infection with *H. pullorum*. Two independent biological samples were analysed in quadruplicate. Statistical analyses were performed by Student's unpaired *t*-test using GraphPad Prism software.

Table 5.1 Oligonucleotides used in this study

Gene name	Oligonucleotide sequence (5´ to 3´)	Source
TNF-α	Fw: GGCAGGTCTACTTTGGAGTCATTGC Rv: ACATTCGAGGCTCCAGTGAATTCGG	(32)
IL1-β	Fw: CTACAGGCTCCGAGATGAACAAC Rv: GGGGAACTCTGCAGACTCAAAC	This study
IL-6	Fw: GGGAAATCGTGGAAATGAGAAA Rv: CATTGGAAATTGGGGTAGGAAG	This study
MIP-2	Fw: CCTGATGTGCCTCGCTGTCTG Rv: CCCACCCCCACCCCTTATC	This study
GAPDH	Fw: GAGGGGCCATCCACAGTCTTC Rv: CATCACCATCTTCCAGGAGCG	(32)

5.3 Results

Effect of nitrosative stress agents on *H. pullorum* viability

The viability of *H. pullorum* 6350-92 under NO stress was analysed by exposing cells to different sources of NO. Two NONOates, spermine-NONOate and DPTA-NONOate, which differ in NO releasing rates, and GSNO, which typifies *S*-nitrosothiols and donates a nitroso functional group to another thiol via a transnitration reaction (33), were used. Hence, the bacterial phenotype responses to NONOates and GSNO are expected to be different.

H. pullorum grown to lag phase (OD_{600} ~ 0.05), early exponential phase (OD_{600} ~ 0.2) and stationary phase (OD_{600} ~ 1) (Figure 5.1), in Supplementary Material) was treated with nitrosative stress-generating agents in concentrations that ranged between 50 and 500 μM, and viability was evaluated at several intervals of time up to 84 h (Figure 5.2).

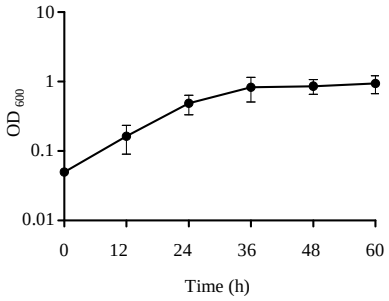


Figure 5.1 Growth of *H. pullorum* cultured under conditions similar to those used in this study. Data are presented as means ± standard deviations of the measured values at an OD600 (n=4).

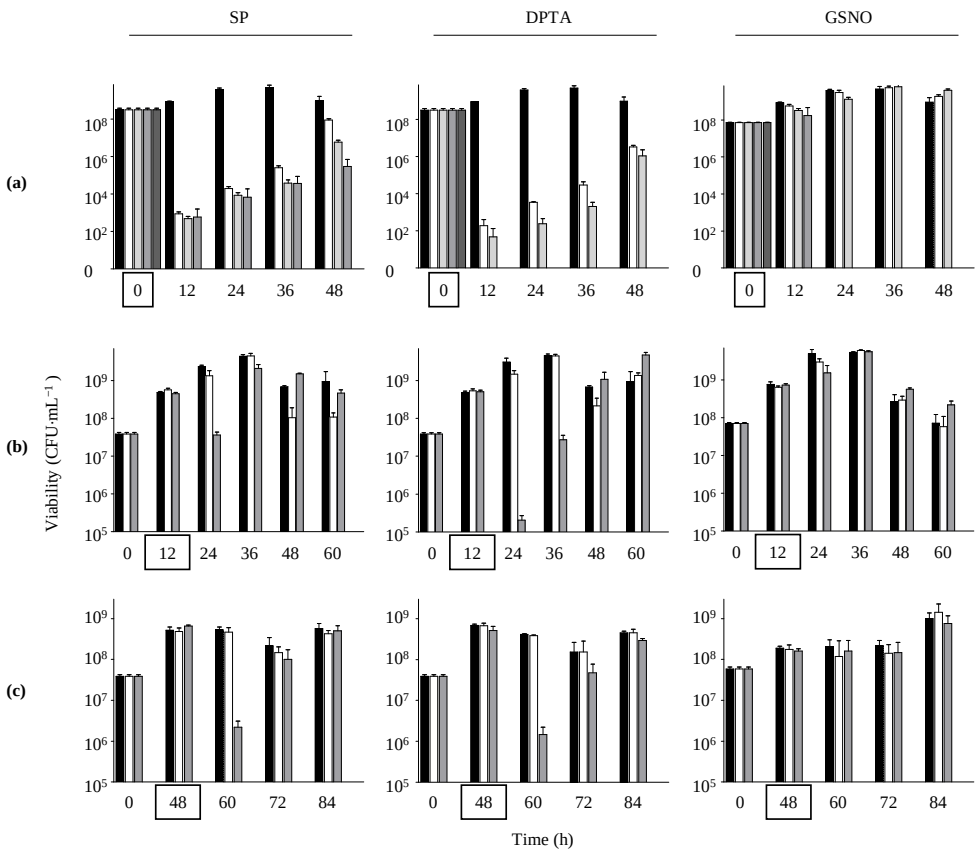


Figure 5.2 Effect of nitrosative stress on *H. pullorum* viability. (a) *H. pullorum* at lag phase ($T = 0$ h), (b) early exponential phase ($T = 12$ h) and (c) stationary phase ($T = 48$ h) was exposed to several concentrations of spermine-NONOate (SP), DPTA-NONOate (DPTA) and GSNO. Nitrosative stress agents were tested at 50 μM (white bars), 100 μM (light grey bars), 250 μM (medium grey bars) and 500 μM (dark grey bars), and black bars depict non-stressed cells. Numbers within squares indicate the time of addition of the NO donor. Data are presented as the mean ± standard deviation of one biological sample with three replicates.

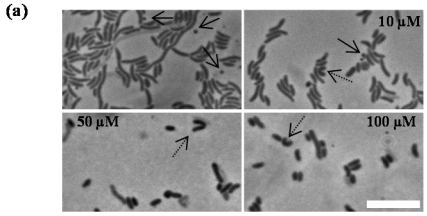
Addition of NONOates to cells at the lag phase caused a viability decrease for all tested concentrations (Figure 5.2a). When cells were treated with the same concentration of NONOate, the inhibitory effect of DPTA-NONOate was higher than

that of spermine-NONOate, which is most probably related to the longer half-life of the former. Nevertheless, in both cases recovery of viability occurred 48h after NO exposure, except for the highest NONOate concentration used (500 μM). Different growth behaviour was observed when the stress was applied to *H. pullorum* at the early exponential phase: for the same concentration, the addition of NONOates to cells at the early exponential phase caused a smaller decrease in viability when compared to cells treated at the lag phase. Moreover, growth recovery to levels similar to untreated cells was observed in a shorter interval of time (Figure 5.2b). The results also showed that no viability impairment occurred when NONOates are applied to *H. pullorum* cells that had reached the stationary phase (Figure 5.2c).

Exposure of lag phase-grown cells of *H. pullorum* to GSNO led to a decrease of viability, but only for high concentrations of GSNO (250 and 500 μM) (Figure 5.2a). The inhibitory effect persisted over time as after 24h treatment the cells exposed to 250 and 500 μM GSNO still displayed very low viability. However, *H. pullorum* did not suffer substantial viability impairment when GSNO was supplied to cells at the early exponential and stationary phases (Figure 5.2b, c). Altogether, it was concluded that NO donors have an antimicrobial action against *H. pullorum* only when cells are at the initial growth phase.

H. pullorum cell size decreases under NO stress

To investigate the morphological alterations caused by nitrosative stress on *H. pullorum*, cells were treated with spermine-NONOate (10-100 μM) and examined by light microscopy. The majority of the *H. pullorum* NO-exposed cells exhibited a bacillary form similar to that observed for untreated cells. Cells with U, V, C and S shapes were also observed, mainly in samples exposed to 50 and 100 μM of spermine-NONOate (Figure 5.3a), as these usually occur when bacteria are changing from bacilli to coccoid form (34-36). Interestingly, exposure of *H. pullorum* to NO stress caused alterations in the cell size, as judged by a decrease in the mean cell size of ~ 25% upon exposure to 50 and 100 μM spermine-NONOate. Moreover, the cell size shortening was accompanied by a decrease in length heterogeneity (Figure 5.3a, b).



SP (µM)	Coccoid (%)	Bacillary (%)
0	1	99
10	2	98
50	5	95
100	2	98

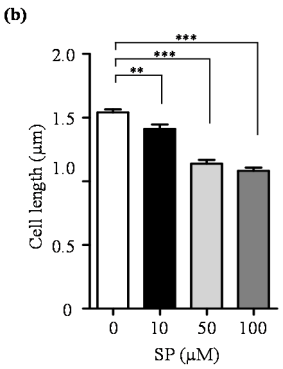


Figure 5.3 Morphology of *H. pullorum* NO-treated cells. (a) Phase-contrast microscopy images, and percentage of coccoid and bacillary forms, of *H. pullorum* cells exposed to the indicated concentrations of spermine-NONOate. Images are representative of the most common morphological phenotype observed for each condition. Morphological forms are indicated as follows: cocci (full arrows) and U/C shape (dashed arrows). Bar, 5 µm. (b) Mean cell length of *H. pullorum* untreated (white bar), and exposed to 10 µM (black bar), 50 µM (light grey bar) and 100 µM (dark grey bar) of spermine-NONOate (SP) for 24 h. Data are presented as the mean ± standard error of the mean. **, $P < 0.01$; ***, $P < 0.0001$ (*t*-test). The percentage of bacillary/coccoid forms and the mean length were determined by manually analysing 100 cells per condition in two independent biological samples.

H. pullorum viability is lower in NO-producing murine macrophages

H. pullorum viability upon infection of J774A.1 murine macrophages was evaluated. When incubated with non-activated macrophages, *H. pullorum* suffered a decrease in survival of less than one log after 5h, and approximately one log after 10h. When in contact with INF-γ/LPS-activated macrophages, *H. pullorum* viability dropped significantly by approximately three logs after 5h, and more than four logs after 10h. Addition to macrophages of the mammalian iNOS enzyme inhibitor L-NMMA (37, 38) rescued *H. pullorum* viability to levels comparable to those observed within non-activated macrophages (Figure 5.4a). Since the release of NO by macrophages killed *H. pullorum*, we next determined the amount of NO produced by

macrophages upon infection. The results show that *H. pullorum* activates the mammalian iNOS as the nitrite content declined to zero when infection occurred in the presence of the iNOS inhibitor L-NMMA (Figure 5.4b). Furthermore, *H. pullorum* viability was inversely related to the amount of NO generated by macrophages as a higher number of *H. pullorum* colonies was observed when lower nitrite contents were present in the supernatants (Figure 5.4). Hence, *H. pullorum* triggers the mammalian innate immune system inducing the iNOS enzyme and the NO generated acts as a bactericidal agent against this pathogen.

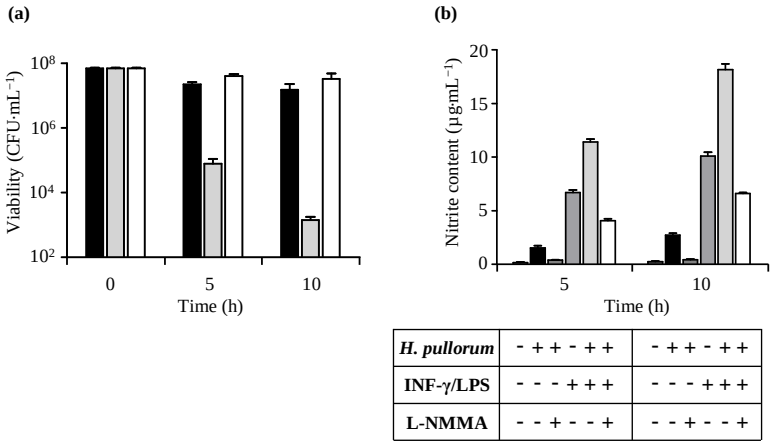


Figure 5.4 *H. pullorum* survival in murine macrophages and nitrite content derived from the NO produced by macrophages. (a) *H. pullorum* viability in J774A.1 murine macrophages: non-activated (black bars), stimulated with INF-γ/LPS (grey bars) and activated in the presence of the iNOS inhibitor L-NMMA (white bars). Bacterial viability was evaluated immediately before infection (time 0), and at 5 and 10 h post-infection. (b) NO produced by macrophages was evaluated in the form of nitrite in the presence (+) and the absence (−) of the indicated components. At least three replicates were analysed for each condition. Data are presented as the mean ± standard error of the mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ (*t*-test).

H. pullorum is phagocytized by murine macrophages

H. pullorum loads in J774A.1 macrophages were quantified by analysis of fluorescent images obtained with confocal microscopy. INF-γ/LPS-activated macrophages that were not infected with bacteria confirmed that stained macrophage cells could be distinguished from stained bacterial cells (Figure 5.5a, b). Bacteria were considered as either internalized or adherent to macrophages based on the simultaneous inspection of three orthogonal slices (Figure 5.5c).

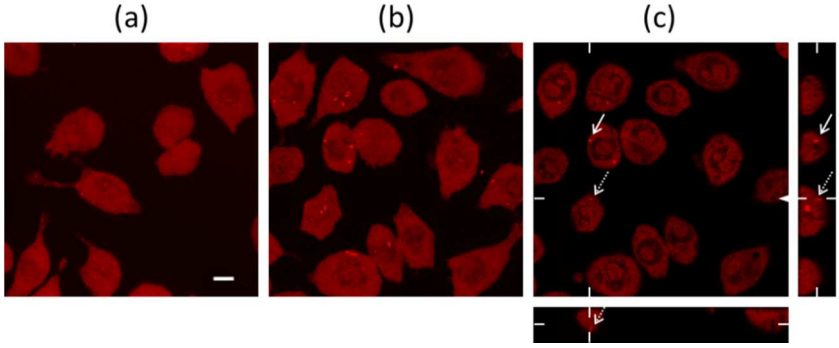


Figure 5.5 Confocal images of macrophages J774A.1 Maximum-intensity projections of whole Z-stacks of (a) non-activated and (b) INF-γ/LPS activated macrophages. In c) orthogonal single planes from a confocal stack of *H. pullorum* infected macrophages is shown. Bacteria location was confirmed by inspection of three planes simultaneously. Bacteria appearing at the surface on, at least 2 orthogonal planes were considered extracellular. Bacteria were treated as internalized when visualized within the cytoplasm in all three orthogonal planes. The dashed and full arrows indicate adherent and internalized bacteria, respectively. Scale bar corresponds to 10 μm.

Phagocytosis of *H. pullorum* by non-activated, activated and iNOS-inhibited macrophages was visualized after incubation periods of 30 min and 2 h (Figure 5.6).

H. pullorum cells were found to adhere to macrophages and to be internalized. The percentage of macrophages with phagocytized bacteria was 70% after 30 min and 86% after 2 h infection. The highest number of bacteria phagocytized per macrophage was observed for activated macrophages, *i.e.* when macrophages were producing significant quantities of NO (Figure 5.6a, c). Concomitantly, suppression of NO production led to a decrease in the number of bacteria per macrophage. In parallel experiments, the viability of *H. pullorum* within macrophages was determined under conditions that replicated those used for confocal microscopy experiments. These results also showed that *H. pullorum* survival was lower in activated macrophages when compared to non-activated and iNOS-inhibited macrophages (Figure 5.6c).

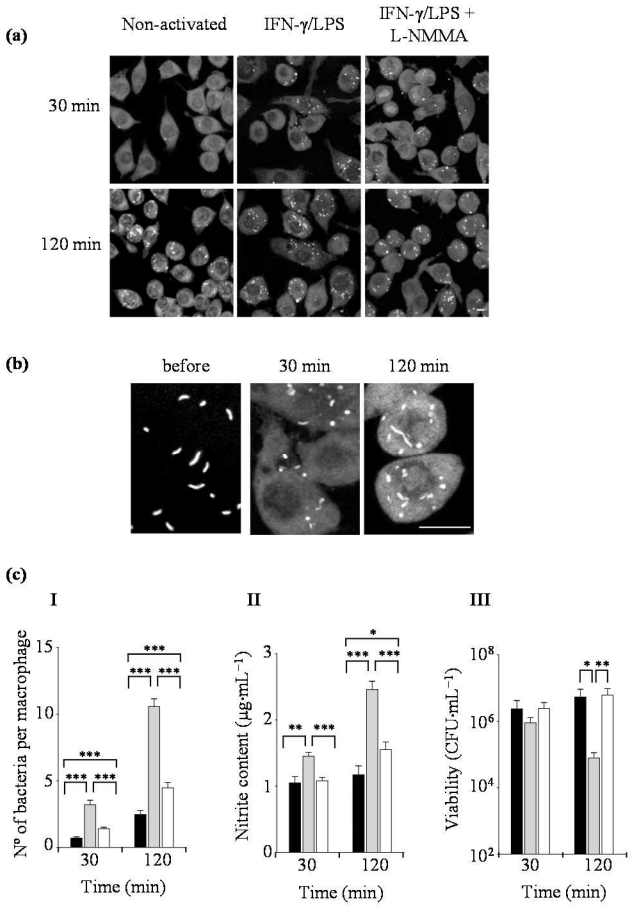


Figure 5.6 Phagocytosis of *H. pullorum* by murine macrophages. (a) Confocal images of *H. pullorum* adhered and internalized by J774A.1 macrophages that were non-activated, or INF-γ/LPS treated in the absence and presence of L-NMMA, and visualized after 30 and 120 min post-infection. (b) Amplified images of *H. pullorum* before and after 30 and 120 min post-infection of INF-γ/LPS-treated macrophages. Confocal images (a, b) are maximum-intensity projections of the whole *Z*-stacks and representative of the most common morphological form observed for each condition for two independent biological replicates. Bars, 10 μm. (c) Mean number of internalized bacteria per macrophage (i), counted from the *Z*-series confocal stacks of a total of 200 macrophages evaluated in two independent biological experiments; and the correspondent macrophage nitrite content (ii) and *H. pullorum* viability (iii) in non-activated (black bars), or INF-γ/LPS-activated macrophages in the absence (grey bars) and presence of L-NMMA (white bars). Data are presented as the mean ± standard error of the mean. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ (*t*-test).

Prior to infection in macrophages, *H. pullorum* cells presented a bacillary shape (Figure 5.6b, before). Upon incubation in macrophages, internalized bacteria exhibited coccoid and shorter bacillary forms (Figure 5.6b, 30 and 120 min). Altogether these results indicate that the production of NO by macrophages increases the phagocytosis of *H. pullorum* cells, which upon internalization acquire a coccoid or shorter bacilli shape.

H. pullorum induces the expression of cytokines in macrophages

J774A.1 macrophages were incubated with *H. pullorum* and the level of gene expression of TNF-α, IL-1β, IL-6 and MIP-2, which has been reported to be linked to iNOS activation (39, 40), was determined. Infection of macrophages with *H. pullorum* for 6 h resulted in the production of IL-1β, TNF-α, IL-6 and MIP-2 cytokines, in relation to non-infected and non-activated macrophages. *H. pullorum* induced the expression of IL-1β, TNF-α, IL-6 and MIP-2 at levels similar to those stimulated by INF-γ/LPS (Figure 5.7).

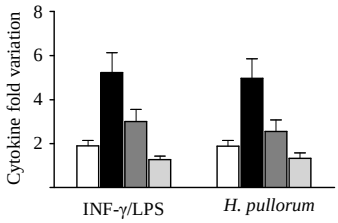


Figure 5.7 Induction of host cytokines by *H. pullorum*. The expression of TNF-α, IL1-β, IL-6 and MIP-2 was quantified, by semiquantitative PCR, in INF-γ/LPS-activated and *H. pullorum*-infected J774A.1 macrophages. For each condition, expression values were quantified by band pixel intensity obtained for the cytokine and normalized to the correspondent band pixel intensity of the constitutive *gapdh* gene. Fold change was determined by dividing values by the normalized cytokine expression in non-infected and non-activated macrophages. Values are presented as the mean ± standard error of the mean of two independent biological samples with at least four technical replicas.

5.4 Discussion

H. pullorum is an emerging human pathogen with zoonotic potential (9), whose mechanisms of pathogenesis and resistance to immunity remain largely unknown. In this work, we have analysed the behaviour of *H. pullorum* when exposed to NO, which is an important antimicrobial chemical weapon of the mammalian immune system. *H. pullorum* is shown to be killed by several NO donors, namely spermine-NONOate, DPTA-NONOate and GSNO, in a growth phase-dependent way. The nitrosative stress strongly impaired *H. pullorum* viability when NONOates were added at the initial growth phase. On the contrary, the nitrosative stress toxicity was negligible for cells that were already at the early exponential and stationary phases. This behaviour was observed even when using very high concentrations of DPTA and spermine-NONOate, *i.e.* at concentrations that completely impair growth when added at the initial growth phase. GSNO is a less efficient bactericide in comparison with NONOates. When *H. pullorum* is at the initial growth phase, GSNO decreased viability only when applied at the highest concentrations. Hence, *H.*

pullorum exhibits a higher resistance to NO stress than *Helicobacter pylori* since it was previously reported that GSNO and DPTA-NONOate significantly impair the growth of *H. pylori* at much lower concentrations (100 and 150 μM, respectively) (41).

Our morphological studies indicated that non-stressed cells of *H. pullorum* grown for 24h retained the bacillary form, with only approximately 1% of the cells presenting a coccoid form. Therefore, coccoid forms of *H. pullorum* seem to occur at latter stages, a result which agrees with previous data showing that after 2 days growth in BHI liquid broth, only 20% of the *H. pullorum* cells exhibited a coccoid form, and that conversion of all cells to the coccoid form occurred after 4 days (42).

The bacillary form of *H. pullorum* cells was not significantly altered upon exposure to nitrosative stress generated by spermine-NONOate at concentrations up to 100 μM. This behaviour of *H. pullorum* apparently contrasts with that of *H. pylori*, as the latter was reported to undergo a rapid conversion from bacillary to the coccoid form when exposed to NO donors (34, 43).

Although NO did not induce the appearance of coccoid forms *in H. pullorum*, the stress led to a decrease in the mean length of the bacteria. A decrease in bacterial size due to environmental factors is not a very common phenomenon, but it was reported for *Escherichia coli* when entering the stationary phase and for marine bacteria during starvation. For marine bacteria, cell size shortening has been proposed to represent a survival mechanism that facilitates nutrient acquisition (44).

Incubation of *H. pullorum* with NO-generating murine macrophages lowered the viability of the bacterium, and the macrophage-killing ability was dependent on the NO produced as treatment of macrophages with an iNOS inhibitor increased the survival of internalized *H. pullorum* cells. The NO-producing murine macrophages phagocytized a higher number of *H. pullorum* cells that rapidly became non-viable. Interestingly, *H. pylori* was reported to increase the expression of iNOS by stimulating the number of macrophages and lymphocytes in the gastric mucosa, and nitrosative stress killed the bacterium (45, 46). However, to the best of our knowledge, the increase of phagocytized cells by NO-producing murine macrophages has not yet been reported for any *Helicobacter* spp.

Our work indicates that *H. pullorum* adheres to murine macrophages, as also noted by (47). Furthermore, we report, for what is believed to be the first time, that *H. pullorum* is internalized in murine macrophages and induces the secretion of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and MIP-2. Similarly, *H. pylori* and *Helicobacter hepaticus* infections have been reported to induce IL-1β, TNF-α and IL-6 (48-50). Moreover, our observation that in murine macrophages *H. pullorum* increases the expression of MIP-2, the IL-8 homologue gene, is consistent with previous studies of *H. pullorum* done in human gastric and intestinal epithelial infected cell lines showing IL-8 induction (22).

The induction of NO and pro-inflammatory cytokines in macrophages by *H. pullorum* observed in this study may be associated with the interaction of macrophage receptors with *H. pullorum* LPS, possibly through activation of NF-*κβ*, similarly to the activation of IL-8 by *H. pullorum* previously observed in epithelial cells (22). Furthermore, the ability here shown to elicit inflammatory responses in a mammalian host suggests that *H. pullorum* can be pathogenic for humans.

5.5 Acknowledgements

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5.6 References

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Chapter VI

The nitrosative stress defences of the enterohepatic *Helicobacter pullorum*

This chapter is part of a manuscript in preparation:

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Adelina Margarida Parente was involved in all the work, having done all experiments except the EPR and peroxynitrite reduction assays. Adelina Margarida Parente also participated in the preparation of the manuscript.

Summary

Helicobacter pullorum is an enterohepatic species infecting animals and humans that stimulates the mammalian innate immunity to produce antimicrobial reactive nitrogen species. The genome of *H. pullorum* was examined and two haemoglobins and three peroxiredoxin-like genes putatively involved in nitrosative stress defence were identified. The expression of these genes was analysed under nitrosative stress conditions. Strains deleted in these genes were constructed and growth and viability was evaluated upon exposure to nitrosative stress. The contribution of these systems to the *H. pullorum* infection of murine macrophages was assessed. All proteins were recombinantly produced and characterized.

We show that *H. pullorum* contains a single domain and a truncated haemoglobin that are induced by the nitric oxide donor nitrosogluthatione and complement the nitrosative stress sensitive phenotype of an *E. coli* flavohaemoglobin mutant. The *H. pullorum* single domain globin provided *in vitro* resistance to nitrosative stress and improved the ability of *H. pullorum* to survive when contacting host mammalian cells, such as macrophages. Deletion of the *H. pullorum* truncated globin did not change the sensitivity to nitrosative stress and macrophage killing. The purified single domain and a truncated haemoglobins contain haem *b* as its cofactor, and present, respectively, a low-spin and a high-spin iron in its ferric state.

The peroxiredoxin-like genes of *H. pullorum*, named as peroxiredoxins one, two and three (*prx1*, *prx2* and *prx3*), are homologs of the bacterial peroxiredoxins bacterioferritin comigratory protein, alkyl hydroperoxide reductase and thiol peroxidase, respectively. Prx2 was shown to be induced in response to peroxynitrite and Prx3 in response to hydrogen peroxide. Whereas the *prx1* mutant did not show increased sensitivity to oxidative and nitrosative stresses and to macrophage killing, the *prx2* mutant presented a growth defect even under normal growth conditions. The construction of the *prx3* mutant was not possible, which suggests that the gene has an essential role in *H. pullorum*. The three recombinant *H. pullorum* peroxiredoxin proteins were shown to detoxify peroxynitrite, having Prx3 the highest activity.

Altogether the results show that the single domain globin and the peroxiredoxins contribute to the nitrosative stress protection of *H. pullorum*.

6.1 Introduction

Helicobacter pullorum is an enterohepatic *Helicobacter* species that colonizes the gastrointestinal tract of birds (1-4), mice (5, 6), rats (7, 8) and humans (1, 9, 10). The bacterium has been described as a potential human pathogen due to its isolation from human with digestive disorders and bacteraemia (1, 9-14). *H. pullorum* infection is suggested to occur via a type-VI secretion system (15), and the induction of the host cell cytotoxicity is mediated by the bacterial cytolethal distending toxin (CDT) activity (16-18). *H. pullorum* was reported to trigger the host immunity, since the bacterium induces IL-8 secretion through the NF-κβ pathway on epithelial cells (19, 20) and *H. pullorum* CDT was shown to induce the expression of genes involved in a Th17 inflammatory response (20). We have shown that *H. pullorum* internalizes and activates murine macrophages, and stimulates the expression of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and MIP-2. Additionally, the nitric oxide (NO) produced by mammalian macrophages was seen to decrease *H. pullorum* viability (Chapter V). Release of NO and superoxide by mammalian inducible nitric oxide synthase (iNOS) and NADPH oxidase (NOX) enzymes, respectively, is a mechanism of the innate immunity for bacterial clearance. Within the phagolysosome the pathogen is exposed to these compounds and their derivatives that known as reactive oxygen and nitrogen species (ROS and RNS), which include, for example, hydrogen peroxide and peroxynitrite (21). In *H. pullorum*, the systems used to avoid the damage caused by these toxic molecules remain to be elucidated.

In general, the resistance of bacteria to NO and RNS has been related to the presence of flavodiirons and haemoglobins (22-38). Haemoglobins are a diversified group of globin-like haem proteins that includes flavohaemoglobin, single domain haemoglobin, and truncated haemoglobin. Flavohaemoglobins present a two-domain structure composed by a N-terminal haem-globin fused with a C-terminal

reductase domain that binds NADPH and FAD. In a large number of bacteria, flavohaemoglobin is a major NO-detoxifying enzyme that protects against nitrosative stress, under aerobic and anaerobic conditions (22). Single domain haemoglobins lack the reductase domain, present in flavohaemoglobins and their role in NO protection has been studied in *Vitreoscilla stercoraria* (Vhb) and *C. jejuni* (Cgb) (26, 28, 30, 39). A chimeric protein comprising Vhb fused with a flavoreductase domain of the *Ralstonia eutropha* flavohaemoglobin was described to detoxify NO, and provided to *E. coli* protection against nitrosative stress (30). The *C. jejuni cgb* mutant strain exhibited markedly inhibition of the aerobic respiration and hypersensitivity to NO and other nitrosating agents (26, 28, 39).

Truncated haemoglobins, or 2-on-2 haemoglobins, are 20-40 residues shorter than single domain haemoglobins and have a different α-helical fold. Truncated haemoglobins are divided in three groups, I, II and III, or N, O and P, respectively. Although the function of truncated haemoglobins is not fully understood, the proteins have been proposed to be involved in oxygen and NO metabolism (40). In particular, the role of the truncated haemoglobins belonging to groups I and II was studied in the cyanobacterium *Synechococcus* spp. PCC 7002, *Mycobacterium* species and *Pseudoalteromonas haloplanktis*. The group I truncated haemoglobin GlbN of *Synechococcus* spp. PCC 7002 protects the bacterium against NO and high levels of nitrate (41). *M. tuberculosis* group I truncated haemoglobin HbN, encoding *glbN* gene, presents NO activity and enhances the heterologous survival of *S. typhimurium* within macrophages (31). The expression of the *M. tuberculosis glbN* in an *E. coli* flavohaemoglobin (*hmp*) mutant, under aerobic conditions, restored the resistance to NO (32). In *M. bovis*, HbN detoxifies NO to nitrate, and the *glbN* mutant strain has impaired ability to metabolize NO and lower NO respiration rates (23). *M. leprae* group II truncated haemoglobin (HbO), the only haemoglobin apparently present in the genome, was shown to be involved in both hydrogen peroxide, NO and peroxynitrite scavenging (33-35). As for *M. leprae* HbO, the group II truncated haemoglobin Ph-2/2HbO of *P. haloplanktis*, encoded by the *PSHAa0030* gene, was reported to catalyse peroxynitrite, *in vitro*. In addition, Ph-2/2HbO conferred resistance to *P. haloplanktis* against oxidative and

nitrosative stresses and restored the growth and cellular respiration of an *E. coli hmp* mutant under nitrosative stress conditions (36, 37).

Whitin group III, the truncated haemoglobin from *C. jejuni* (Ctb) and *H. hepaticus* (HbP) are the best biochemically characterized proteins (42-44), but their contribution to NO protection was so far only investigated for Ctb. Data showed that even though *ctb* is up-regulated in response to nitrosative stress, the protein seems not to contribute to *C. jejuni* NO protection (38, 45).

Peroxiredoxins are a group of ubiquitous proteins that are usually described to confer resistance to oxidative stress. These enzymes detoxify hydrogen peroxide and a wide range of organic hydroperoxides (46-52). In *H. pylori*, *M. tuberculosis* and *S. typhimurium*, peroxiredoxins were described to contribute to peroxynitrite reduction (53-55). Peroxiredoxins are classified into typical 2-Cys, atypical 2-Cys, and 1-Cys based on the number and location of their catalytic cysteines. More recently, peroxiredoxins were divided into six subfamilies, based in their amino acid similarity (56, 57), being the alkyl hydroperoxide-reductase (AhpC)/Prx1 (46, 50, 51, 53, 54, 58, 59), thiol peroxidase (Tpx) (48-50, 54) and the bacterioferritin comigratory protein (Bcp)/PrxQ (47, 48, 52) the most studied peroxiredoxin subfamilies in the bacterial kingdom. All peroxiredoxins share the same basic catalytic mechanism, in which an active-site cysteine (the peroxidatic cysteine, C_P) is oxidized to a sulfenic acid by the peroxide substrate forming a disulfide with another cysteine residue (the resolving cysteine; C_R) (57).

Although the increasing evidence for the potential proinflammatory and pathogenic roles of *H. pullorum* in humans, the molecular mechanisms of this bacterium to fight host defences remain unaddressed. The aim of this work was to study homologs of haemoglobins and peroxiredoxins in *H. pullorum* and elucidate whether they contribute to protect this emergent pathogen against oxidative and nitrosative stresses.

6.2 Materials and Methods

Analysis of *H. pullorum* genome

Search of *H. pullorum* genes encoding homologs of bacterial haemoglobins and peroxiredoxins were done on the genome of the *H. pullorum* MIT 98-5489 (available at the gene bank, assembly accession GCA_000155495.1, project ABQU00000000.1). Protein-protein BLAST algorithm at NCBI BLAST was used for amino acid sequence similarities studies, and alignments were performed with Clustal X 2.1 version and edited with the Genedoc software.

Bacterial strains, plasmids and growth conditions

All strains and plasmids used in this study are listed in Table 6.1. *Helicobacter pullorum* 6350-92 (CCUG 33838), isolated from a stool sample of a patient with gastroenteritis and hepatitis (60), was used as the wild type strain. Cells were routinely cultivated at 37 °C in a microaerobic atmosphere (6% O_2, 7% CO_2, 3.5% H_2, and 83.5% N_2) generated by the Anoxomat system (Mart Microbiology), in horse blood agar (BA) composed of Blood Agar Base no. 2 (Oxoid) with 10% (v/v) defibrinated horse blood (Probiológica), supplemented with an antibiotic-antifungal mix composed by 6.3 $g \cdot L^{-1}$ vancomycin (Roth), 3.1 $g \cdot L^{-1}$ trimethoprim (Sigma) and 2.5 $g \cdot L^{-1}$ amphotericin B (Roth), and, when required, 20 $\mu g \cdot mL^{-1}$ of kanamycin or 5 $\mu g \cdot mL^{-1}$ of gentamicin. Bacteria were taken as fully grown when cultured in BA plates for 5 days, with two intermediate plating passages.

E. coli pre-cultures were grown overnight at 37 ºC and 150 r.p.m. on Luria-Bertani (LB) medium that, when required, were supplemented with kanamycin, ampicillin, and isopropyl-1-thio-β-d-galactopyranoside (IPTG).

Table 6.1 Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Source
Strains		
***H. pullorum* 6350-92**	Parental strain	(60)
Δ*trHb*	HPMG_00979 deletion mutant; Km^r	This study
Δ*sdHb*	HPMG_00954 deletion mutant; Km^r	This study
Δ*trHb*Δ*sdHb*	HPMG_00979 and HPMG_00954 deletion mutant; Km^r Gm^r	This study
Δ*prx1*	HPMG_00817 deletion mutant; Gm^r	This study
Δprx*2*	HPMG_00739 deletion mutant; Km^r	This study
Δ*prx1*Δ*prx2*	HPMG_00817 and HPMG_00739 deletion mutant; Km^r Gm^r	This study
E. coli		
K-12 ATCC 23716	Parental strain	ATCC
Δ*hmp*	LMS2552; K-12 *hmp* mutant; Km^r	(61)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacIq* ZΔM15 Tn*10*]; Tet^r	Agilent Technologies
BL21 Gold (DE3)	F^- *ompT hsdS*(r_B^- m_B^-) dcm^+ *galλ endA* Hte; Tet^r	Stratagene
Plasmids		
pFLAG-CTC	Vector for protein expression under the influence of the *tac* promoter; Amp^r	Sigma
pFLAG-CTC-*trHb*	pFLAG-CTC carrying the *trHb* gene subcloned into NdeI and XhoI sites; Amp^r	This study
pFLAG-CTC-*sdHb*	pFLAG-CTC carrying the *sdHb* gene subcloned into NdeI and XhoI sites; Amp^r	This study

Real-time qRT-PCR analysis

Fully grown *H. pullorum* cells were inoculated in 25 cm^2 cell culture flasks (Nunc) filled with 10 mL Brain Heart Infusion (BHI) broth (Oxoid) plus 10% defibrinated Fetal Calf Serum (FCS, Gibco-Invitrogen) (BHI-FCS), to an initial optical density at 600 nm (OD_{600}) of 0.1-0.2. Cells were grown for 19 h, at 150 r.p.m. in microaerobic conditions, and these cultures were used to inoculate in fresh BHI medium supplemented with 0.2% (v/v) β-cyclodextrin (Sigma), (BHI-βCD) at an OD_{600} of 0.1. When cells reached an OD_{600} of ~ 0.5, 100 µM of the nitrosative stress generator S-nitrosogluthatione (GSNO), which was freshly prepared by mixing equimolar amounts of sodium nitrite and reduced glutathione under acidic conditions (0.05 M HCl) (62), or 50 µM of hydrogen peroxide (Panreac) were

added. After 30 min or 1 h of incubation with hydrogen peroxide and GSNO, respectively, a mixture of ethanol:phenol (95:5) was added to stabilize the RNA and cells were collected by centrifugation (10 min, 8000 ***g***, 4 ºC). As peroxynitrite reacts quickly with BHI medium, *H. pullorum* was firstly grown as described above but using Brucella Broth (Oxoid, BB) containing 5% FCS instead of BHI-FCS and BHI-βCD and treated with 50 μM peroxynitrite (Cayman), for 15 min.

Total RNA was then isolated with the High Pure RNA Isolation kit (Roche), and the residual DNA was removed by treatment with Turbo DNA-free (Ambion). RNA was quantified in a NanoDrop spectrophotometer (Thermo Scientific), and its integrity confirmed by agarose gel electrophoresis. For each sample, 200 ng RNA was converted to cDNA using the anchored-oligodT primers and the Transcriptor High Fidelity cDNA Synthesis kit (Roche).

Real-time PCR experiments were done according to the manufacturer's instructions in a LightCycler Instrument using LightCycler FastStart DNA Master SYBER Green I Kit (Roche Applied Science). The amplification reactions were carried out with equal amounts of cDNA (300 ng) as initial template, and each reaction contained 0.5 μM of the specific primers (Table 6.2), 4 mM of $MgCl_2$, and the hot-start PCR reaction mix from Roche Applied Science. The expression ratio of the target gene was determined relatively to a reference gene, the *H. pullorum* DNA gyrase subunit A gene (*gyrA*, HPMG_00492) (63) that does not change expression under these conditions. At least two biological samples were analysed in duplicate.

Table 6.2 Primers used in this study

Primer designation	Oligonucleotide Sequence (5' to 3')
Quantitative real-time RT-PCR	
gyrA_RT_fw	GAGGCTTATGAAACAGGGAGAGG
gyrA_RT_rv	CATAGGGGTTGATTTAAAAAGGTGA
TrHb_RT_fw	TGTGTGCCTCCCACTCTATATCG
TrHb_RT_rv	ATGGGCTTGGCGAGATTTT
SdHb_RT_fw	GAGTGTTTGCTTGTGGCGATT
SdHb_RT_rv	CCACGCCTCTAGCACTTCATC
Prx1_RT_fw	TAACCCCCAAGCACCATAAG
Prx1_RT_rv	GAGGAAGTTGGGTAGTTTTGTATTT

Table 6.2 Continuation

Primer designation	Oligonucleotide Sequence (5' to 3')
Prx2_RT_fw	CAACCAGCAGGGCAAACTT
Prx2_RT_rv	ACTCCGGTAAATCAAGGTGGTAT
Prx3_RT_fw	GCGAACCTGCCAAAATCACAC
Prx3_RT_rv	TAGGCGGAGCAAGTGGGAAGT
Gene deletion	
TrHb_A1	CTTGCATTTCCAAAATCCTAACCAT
TrHb_A2	TTATTCCTCCTAGTTAGTCAATAAAATCTCCTTATAAATAAAATGC
TrHb_B1	TACCTGGAGGGAATAATGAGCTAAAGTGGTGGTAAATTTACCTCAATCTATCC
TrHb_B2	GCGGGTATCCTGATTGTAGAGAGATTTTTTATC
SdHb_A1	CCTACAAACTTCTGTCCATCAAATCTAATAAGGGGCTG
SdHb_A2	TTATTCCTCCTAGTTAGTCAAATAAATCCTTTTGACTTCA
SdHb_B1	TACCTGGAGGGAATAATGATTGATTTGCAAAAAAACTT
SdHb_B2	GTATGAGGGAGGCTTGGCGACAATGCGTTATTCTATC
Prx1_A1	TAAAGGAGAGCAAATAGAGCA
Prx1_A2	TTATTCCTCCTAGTTAGTCACTTATATCCTTGTTAAGTTTT
Prx1_B1	TACCTGGAGGGAATAATGAAAGAATTGCAAGGCTAACT
Prx1_B2	TCTATTGTCTTTGGAGGAA
Prx2_A1	ATATCGGGGCTTTGTTCTC
Prx2_A2	TTATTCCTCCTAGTTAGTCATTTGGAGCTTTTTTTGTAAC
Prx2_B1	TACCTGGAGGGAATAATGATCGCATTCTTCTATCTTCCC
Prx2_B2	CAAATACTCTTGTGCCGTGT
Cassette amplification	
C1	GGAATTGTGAGCGGATAAC
C2	CCAGTCACGACGTTGTAAA
Gene cloning	
TrHb_NdeI	GGAGATCATATGCAATATCAAGAAA
TrHb_XhoI	GTTCCAAAGGATAGCTCGAGGTAAA
SdHb_NdeI	TTTCATATGTTAGATATACAAAC
SdHb_XhoI	AAGATCTCGAGAAAATCAAAAA
Prx1_NdeI	CATATGGAATTGCAAATTGGAGAT
Prx1_XhoI	CAAACTCGAGAAAGAAGTGCTAAC
Prx2_NdeI	GGATTTCATATGTTAGTTAC
Prx2_XhoI	GAAGCCTCGAGCAACTTAG
Prx3_NdeI	TTAATAAGGATAAACATATGGTTACTT
Prx3_XhoI	TTCTATCTAAACTCGAGCAACAC
N26	CATCATAACGGTTCTGGCAAATATTC
C24	CTGTATCAGGCTGAAAATCTTCTC

Construction of *H. pullorum* mutants

The nonpolar truncated haemoglobin (*trHb*), single domain haemoglobin (*sdHb*), peroxiredoxin 1 (*prx1*) and peroxiredoxin 2 (*prx2*) single deletion mutants (Δ*trHb*, Δ*sdHb*, Δ*prx1* and Δ*prx2*; Table 6.1), were constructed by inactivation of the *H. pullorum* HPMG_00979, HPMG_00954, HPMG_00817 and HPMG_00739 genes, which was achieved by allelic exchange after transformation of *H. pullorum* with a three-fragment assembly product that consisted of the flanking regions of the target gene and the kanamycin *aphA-3* or the gentamycin *aac(3)-IV* (64) cassettes. To this end, a region upstream of each target gene was PCR amplified with Phusion polymerase (Thermo Scientific) and oligonucleotides A1/A2 (Table 6.2), a region downstream of each target gene was amplified with oligonucleotides B1/B2 (Table 6.2), using the genomic DNA of *H. pullorum* 6350-92 as template, while the resistance cassettes previously cloned in pUC18 were amplified with primers C1/C2 (Table 6.2). Then, the three DNA fragments were assembled by PCR using oligonucleotides A1 and B2.

The three-fragment DNA assembly products were introduced into *H. pullorum* 6350-92 by electroporation (65). Briefly, *H. pullorum* cells were harvested from two fully grown BA plates, resuspended in 2 mL ice-cold washing buffer (270 mM sucrose, 15% glycerol), and 3 times washed (10 min, 10,000 ***g***, 4 ºC) and resuspended in 1/10 volume of washing buffer. 50 mL of a cell suspension were electroporated with 1-2 µg DNA, using a MicroPulser (Bio Rad) at 1.80 kV, 200 V, and 25 F. Immediately after electroporation, 200 µL recovery broth (BHI, 10% glycerol, 10% FCS) was added and cells were plated on non-selective BA plates containing the antibiotic cocktail (above described). After an overnight incubation, cells were plated on selective BA plates with 20 $\mu g \cdot mL^{-1}$ kanamycin or 5 $\mu g \cdot mL^{-1}$ gentamicin. The presence of the desired mutations in the *H. pullorum* chromosomal DNA was confirmed by PCR analysis.

The *trHb* and *sdHb* double deletion mutant (Δ*trHb*Δ*sdHb*; Table 6.1), was constructed by the introduction of the three-fragment assembly product mentioned above, which carried the gentamycin cassette and the upstream and downstream *trHb* regions, in the Δ*sdHb H. pullorum* strain. The *prx1* and *prx2* double mutant

(Δ*prx1*Δprx2; Table 6.1) was constructed by electroporation of the three-fragment assembly product that carried the kanamycin cassette within the flanking fragments of *prx2* in the Δ*prx1 H. pullorum* strain.

Growth assays

H. pullorum wild type and haemoglobin mutants were exposed to GSNO, and the wild type and the peroxiredoxin mutants were exposed to hydrogen peroxide and peroxynitrite, or left untreated (control).

To this end, 19 h grown cultures prepared as described above for the Real-time PCR protocol were reinoculated in BHI-βCD medium to an OD_{600} of 0.1. When cells reached an OD_{600} ~ 0.4, 100 µM GSNO was added. For the hydrogen peroxide susceptibility experiments, the 19 h grown cultures were reinoculated in BHI-βCD to an OD_{600} of 0.1 and distributed into 24-well plates, with or without the addition of 1 and 5 mM of hydrogen peroxide. Growth was measured by measuring OD_{600} for 10 or 24 h.

For the peroxynitrite assays, *H. pullorum* cells firstly grown as described before immediately above but using BB plus 2.5% FCS (instead of BHI-FCS and BHI-βCD) and reinoculated in 10 mL medium to an OD_{600} of 0.05. Peroxynitrite (50 µM) was added to the cultures and growth and viability was monitored after 2, 7 and 11 h by measuring the OD_{600} and by serial diluting each sample in BHI and plating on antibiotic-antifungal cocktail containing BA plates. The number of viable cells (colony-forming units ($CFU \cdot mL^{-1}$) was evaluated upon incubation in BA plates, for 48 h.

Macrophages assays

Murine macrophages J774A.1 (ATCC TIB-67) were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose, GlutaMAX™ Supplement, pyruvate (GIBCO), 10% FCS and 100 $\mu g \cdot mL^{-1}$ penicillin/streptomycin (Sigma), and incubated in a humidified 5% CO_2-air controlled atmosphere, at 37 ºC.

Fully grown *H. pullorum* was used to inoculate fresh BA plates that grown for 24 h, which were next used to inoculate, at an OD 0.1-0.2, BB medium containing 5% FCS, and grown for 15 h. Immediately before infection, *H. pullorum* cells were pelleted (10 min, 8740 ***g***, 4 ºC), resuspended in the infection medium Roswell Park Memorial Institute 1640 Medium (RPMI), with GlutaMAX™ Supplement (GIBCO) containing 10% FCS (RPMIi) at an OD_{600} of 0.2, and the bacterial viability determined by CFU counting prior to the incubation within macrophages (time zero of infection).

For the infection assays with *H. pullorum* wild type and the mutant strains, macrophages were seeded overnight in 24-well plates at a concentration of 2 x 10^5 cells/well. Macrophages were then activated for 5 h with 0.15 $\mu g \cdot mL^{-1}$ gamma interferon (INF-γ, Sigma) and 0.5 $\mu g \cdot mL^{-1}$ lipopolysaccharides (LPS, Sigma). When required, murine iNOS activity was inhibited with 800 μM L-NMMA, which was added simultaneously to INF-γ and LPS. Immediately before infection, the macrophage medium was changed to the infection medium RPMIi and, where indicated, supplemented with the L-NMMA inhibitor. Macrophages were infected with *H. pullorum* wild type and the mutant strains at a multiplicity of infection (MOI) of 100. After 2 and 6 h of incubation the supernatants were collected for the quantification of nitrites by the Greiss method (66). Each well was washed three times with PBS, and macrophages were lysed with 2% saponin (w/v) and their bacterial content evaluated by CFU counting.

Complementation assays

For over-expression of each of the *H. pullorum* haemoglobins in the *E. coli* flavohaemoglobin mutant LMS2552 (Δ*hmp*) (61), the HPMG_00979 and HPMG_00954 genes were PCR amplified with Phusion polymerase from the genomic DNA of *H. pullorum* 6350-92, using the primer pairs TrHb_NdeI/TrHb_XhoI and SdHb_NdeI/SdHb_XhoI (Table 6.2). The resultant DNA fragments were cloned in digested NdeI/XhoI plasmid pFLAG-CTC, yielding the pFLAG-*trHb* and pFLAG-*sdHb* plasmids. Competent cells of *E. coli* wild type and Δ*hmp* were transformed with pFLAG-CTC, pFLAG-*trHb* and pFLAG-*sdHb*.

For the mutant strain, the positive transformants were selected on LA medium containing 100 $\mu g \cdot mL^{-1}$ ampicillin plus 25 $\mu g \cdot mL^{-1}$ kanamycin, while for the *E. coli* wild type selection was done with ampicillin (100 $\mu g \cdot mL^{-1}$). Positive colonies were screened by PCR using the primers N26 and C24 from Sigma (Table 6.2).

Overnight pre-cultures of *E. coli* wild type and Δ*hmp* carrying the empty pFLAG, pFLAG-*trHb* and the pFLAG-*sdHb* were grown in LB supplemented with the respective antibiotics and 1 mM IPTG, and used to inoculate fresh LB medium supplemented with 12 µM $FeCl_3$, 1 mM aminolevulinic acid and 1 mM IPTG to an initial OD_{600} of 0.05. Cells were grown aerobically for 8 h, untreated or exposed to 200 µM GSNO, and OD_{600} was monitored at 1 h intervals.

Production of recombinant haemoglobins and peroxiredoxins of *H. pullorum*

Haemoglobins and peroxiredoxins homolog genes of *H. pullorum* 6350-92 were amplified with Phusion polymerase and primers based on flaking sequences that generated NdeI and XhoI restriction products (Table 6.2) with 436 bp (*trHb*), 563 bp (*sdHb*), 506 bp (*prx1*), 628 (*prx2*) and 535 (*prx3*) bp. These genes were cloned into NdeI/XhoI digested expression vector pET28a+ (Novagen) to produce proteins with a poly-histidine tail fusion (His-tag) at the N-terminal region, and transformed in *E. coli* XL1 Blue. Positive recombinant plasmids were selected from kanamycin (30 $\mu g \cdot mL^{-1}$) resistant colonies in Luria-Bertani Agar (LA) medium, and the integrity of the genes was confirmed by gene sequencing.

Over-expression of the recombinant proteins was achieved in *E. coli* Bl21Gold (DE3), which was grown aerobically in LB supplemented with 30 $\mu g \cdot mL^{-1}$ kanamycin at 37 ºC and 150 r.p.m. For the over-expression of haemoglobins, the broth was also supplemented with 8 $mg \cdot L^{-1}$ Fe (in the form of $FeSO_4.7H_2O$). When cells reached OD_{600} of 0.8, 400 µM IPTG was added and cells were further incubated overnight at 20 ºC and 150 r.p.m. Cells were harvested by centrifugation (10 min, 8000 ***g***, 4 ºC), resuspended in Tris-HCl 20 mM, disrupted in a French pressure cell, and ultracentrifuged at 100,000 ***g*** and 4 °C for 2 h. The soluble

fractions were loaded into a charged nickel-IMAC Sepharose HP column (GE Healthcare) equilibrated with buffer Tris-HCl 20 mM (pH 7.5) and 500 mM NaCl and eluted in the same buffer that contained 500 mM imidazole. After overnight dialysis of the peroxiredoxins against 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl, and of the haemoglobins against 20 mM Tris-HCl (pH 7.5), 250 mM NaCl and 20% glycerol, the protein size and purity was evaluated by SDS/PAGE with the Roti-Mark protein molecular mass marker (Carl Roth). The protein concentration was determined with the biocinchoninic acid assay (67). Haem quantification and determination was done by the pyridine haemochrome method (68).

UV–Visible spectroscopy and EPR

UV–Visible absorption spectra of *H. pullorum* proteins were acquired at room temperature using a Shimadzu UV-1700 spectrophotometer. Electron paramagnetic resonance (EPR) spectra of *H. pullorum* haemoglobins were obtained on a Bruker EMX spectrometer, equipped with an Oxford Instruments continuous flow helium cryostat. The EPR spectra were recorded at 12 K, at 9.4 GHz, microwave power of 2 mW and modulation amplitude of 1 mT.

Peroxynitrite assays

The reaction of peroxynitrite ($ONOO^-$, ε_{302} nm = 1.67 x 10^3 $M^{-1} \cdot cm^{-1}$) with the reduced proteins Prx1, Prx2 and Prx3 from *H. pullorum* was investigated by time-resolved spectroscopy, using a thermostated stopped-flow instrument (DX.17MV, Applied Photophysics) equipped with a 1-cm path length observation chamber. Experiments were carried out according to the 'initial rate approach' (69). The proteins were reduced by a 2 h incubation with 10 mM dithiothreitol (DTT) at room-temperature. Prior to the experiments, the excess DTT was removed and the buffer exchanged to 100 mM phosphate buffer pH = 7.0 plus 0.2 mM diethylenetriamine pentaacetic acid (DTPA) by concentration/dilution cycles. Afterwards, the proteins were gently degassed and anaerobically mixed in a 1:1 ratio at increasing concentrations (from 5 to ~ 200 µM) with a solution of 40-50

µM peroxynitrite in 10 mM NaOH. The reaction was investigated at 5 °C. The initial rate of peroxynitrite decomposition (V_o) was obtained from the absorption decrease measured at 310 nm, using $\varepsilon = 1.6 \times 10^3\ M^{-1}\cdot cm^{-1}$ (69). The kinetic traces were analysed from 4 ms on, because a small artifactual signal was invariantly observed over the very first millisecond after mixing. Given the rate law $V_o = k\,[ONOO^-]_0\,[Prx]_0$, the second-order rate constant (k) of the reaction was estimated by linear regression analysis of the dependence of the measured initial rates on protein concentration, and dividing the fitted slope by the initial peroxynitrite concentration ($[ONOO^-]_0$). The concentration of the three proteins was obtained using the following 280 nm extinction coefficients predicted by Bioinformatics Resource Portal of the Swiss Institute of Bioinformatics (http://www.expasy.org/): 24.1 $mM^{-1}\cdot cm^{-1}$, 19.6 $mM^{-1}\cdot cm^{-1}$ and 3.1 $mM^{-1}\cdot cm^{-1}$ for Prx1, Prx2 and Prx3, respectively.

6.3 Results

H. pullorum genome contains two haemoglobin-like encoding genes

Analysis of the *H. pullorum* genome revealed DNA regions coding for two putative haemoglobin-like proteins (HPMG_00954 and HPMG_00979[1]). Their amino acid sequences include the highly conserved histidine residue that participates in haem binding and is present in the position eight of helix F (F8) of all known haemoglobins. Also, other highly conserved residues involved in haem stabilization were found conserved (40, 70) (Figure 6.1).

A more detailed analysis revealed that the coding region of HPMG_00954 shares (56% I/74% S) and (50% I/69% S) amino acid sequence identity/similarity with the single domain haemoglobins *C. jejuni* Cgb and *V. stercoraria* Vhb, respectively. *H. pullorum* single domain haemoglobin (SdHb) shares between 42-48% I/60-67% S with *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *E. coli* flavohaemoglobins. Figure 6.1, highlights the conserved residues of *H. pullorum*

[1] These genes are also nominated as HPMG_RS04780 and HPMG_RS03725.

SdHb when compared with the bacterial single domain haemoglobins and the globin-like domain of flavohaemoglobins.

The *H. pullorum* HPMG_00979 gene product (TrHb) shares amino acid sequence identity and similarity with: i) group III *C. jejuni* Ctb (64% I, and 78% S) and *H. hepaticus* HbP (66% I and 82% S); ii) group I *M. tuberculosis* HbN (29% I and 39% S); and iii) group II HbO (17% I and 35% S) truncated haemoglobins. *H. pullorum* TrHb exhibits several conserved amino acid residues with bacterial group III truncated haemoglobins (Figure 6.1).

```
Hpu_SdHb  : ------------MLDIQTKELVKSTIPALKSQGEDITKVFYRELFTRYPQVKSMFD : 44
Cj_Cgb    : ------------MTKEQIQIIKDCVPILQKNGEDLTNEFYKIMFNDYPEVKPMFN : 43
Vs_Vhb    : ------------MLDQQTINIIKATVPVLKEHGVTITTTFYKNLFAKHPEVRPLFD : 44
Sa_Hmp    : ------------MLTEQEKDIIKQTVPLLKEKGTEITSIFYPKMFKAHPELLNMFN : 44
Pa_Fhp    : ------------MLSNAQRALIKATVPLLETGGEALITHFYRTMLGEYPEVRPLFN : 44
Ec_Hmp    : ------------MLDAQTIATVKATIPLLVETGPKLTAHFYDRMFTHNPELKEIFN : 44
Cj_Ctb    : ------------MKFETINQESIAK---LMEI-------FYEKVRKDKD-LGPIFN : 36
Hpu_TrHb  : ------------MTFEKINVDSIRK---LMDI-------FYAKVRADKSGLGDIFN : 35
Hh_ HbP   : ------------MQYQEICTEAINQ---LMDI-------FYAKIRVDKNGLGEIFN : 36
Mt_ HbN   : ---------MGLLSRLRKREPISIYDKIGGHEAIEVVVEDFYVRVLADDQ-LSAFFS : 43
Mt_HbO    : ------------------MPKSFYDAVGGAKTFDAIVSRFYAQVAEDEV-LRRVYP : 33
Ph_HbO    : MIKRLFSKSKPATIEQTPTPEKTPYEILGGEAGALAIANRFYDIMATDEY-AKPLYD : 52

Hpu_SdHb  : MQKQ----KDGSQPKALAMAVLNAAKNIDNLEKIRPSIESIGKT--HVRLNV-RPEH : 94
Cj_Cgb    : MEKQ----ISGEQPKALAMAILMAAKNIENLENMRSFVDKVAIT--HVNLGV-KEEH : 93
Vs_Vhb    : MGRQ----ESLEQPKALAMTVLAAAQNIENLPAILPAVKKIAVK--HCQAGV-AAAH : 94
Sa_Hmp    : QTNQ----KRGMQSSALAQAVMAAAVNIDNLSVIKPVIMPVAYK--HCALQV-YAEH : 94
Pa_Fhp    : QAHQ----ASGDQPRALANGVLMYARHIDQLQELGPLVAKVVNK--HVSLQV-LPEH : 94
Ec_Hmp    : MSNQ----RNGDQPREALFNAIAAYASNIENLPALLPAVEKIAQK--HTSFQI-KPEQ : 94
Cj_Ctb    : NAIGTSDEEWKEHKAKIGNFWAGMLLGE---GDYN---GQPLKK--HLDLPPFPQEF : 82
Hpu_TrHb  : TKIGTSDEVWEVHKAKIANFWQGMLLNS---GDYN---GQPLKA--HLDLPPFPREL : 85
Hh_ HbP   : NAIGTSDIEWEAHKKKIANFWQGMLLGS---GDYK---GQPLKA--HLDLPPFPREF : 83
Mt_ HbN   : ------GTNMSRLKGKQVEFFAAALGGP---EPYT---GAPMKQV-HQGRGITMHHF : 89
Mt_HbO    : ------EDDLAGAEERLRMFLEQYWGGP---RTYSEQRGHPRLRMRHAP-FRISLIE : 84
Ph_HbO    : ----MHPLPLDRIRQVFFEFLSGWLGGP---DLFVAKHGHPMLRKRHMP-FTIDQDL : 105

Hpu_SdHb  : YPLVGECLLVAIKEVLGA---SDEVLEAWSKAYGEIAEFYIDIEKKIYQEQK----- : 143
Cj_Cgb    : YPIVGACLLKAIKNLLNP---DEATLKAWEVAYGKIAKFYIDIEKKLYDK------- : 140
Vs_Vhb    : YPIVGQELLGAIKEVLGDAA-TDDILDAWGKAYGVIADVFIQVEADLYAQAVE---- : 146
Sa_Hmp    : YPIVGKNLLKAIQDVTGLEE-NDPVIQAWAKAYGVIADVFIQIEKEIYDQMMWIGFK : 149
Pa_Fhp    : YPIVGTCLLRAIREVLGEQIATDEVLEAWGAAYQQLADLLIEAEESVYAASAQADGG : 147
Ec_Hmp    : YNIVGEHLLATLDEMFSP---GQEVLDAWGKAYGVLANVFINREAEIYNENASKAGG : 150
Cj_Ctb    : FEIWLKLFEESLNIVYNEEMKNV----ILQRA-QMIASHFQNMLYKYGGH------- : 127
Hpu_TrHb  : FNVWLNLFEESLRAVYAKEEHISL---ILQRA-QMIAQRFQYIIYESGLHH------ : 127
Hh_ HbP   : FSLWLSLFEECLNKIFSPKIANE----ILQKA-QMIAGRFQYMLYESG-H------- : 130
Mt_ HbN   : SLVAGHLADALTAAGVPSETITE---ILGVIAPLAVDVTSGESTTAPV--------- : 136
Mt_HbO    : RDAWLRCMHTAVASIDSETLDDE---HRRELLDYLEMAAHSLVNSPF---------- : 128
Ph_HbO    : RDQWMYCMNKTLDLEVDNPLLREG---LKQSFGQLASHMINQH------------- : 145
```

Figure 6.1 Amino acid sequence comparison of *H. pullorum* haemoglobins (HPMG_00954 and HPMG_00979) with the best characterized bacterial

haemoglobins. Protein single domain haemoglobin sequences of *H. pullorum* (Hpu_SdHb), *C. jejuni* (Cj_Cgb) and *V. stercoraria* (Vs_Vhb); flavohemoglobins of *S. aureus* (Sa_Hmp), *P. aeruginosa* (Pa_Fhp) and *E. coli* (Ec_Hmp); group III truncated haemoglobins of *C. jejuni* (Cj_Ctb), *H. pullorum* (Hpu_TrHb) and *H. hepaticus* (Hh_HbP); group I truncated haemoglobin of *M. tuberculosis* (Mt_HbN) and group II truncated haemoglobins of *M. tuberculosis* (Mt_HbO) and *P. haloplanktis* (PSHAa0030 gene, Ph_HbO) were aligned using the Clustal X algorithm. Amino acids residues conserved in all single domain haemoglobin and flavohemoglobin sequences are shaded in black and amino acid residues conserved in all group III truncated haemoglobins are marked in grey. The histidine F8 is shaded in red and the other highly conserved residues in blue. The amino acid sequences of flavohemoglobin are not complete.

Expression of the haemoglobins and phenotypic analysis of the *H. pullorum* haemoglobin mutants

The expression of *trHb* and *sdHb* genes in *H. pullorum* treated with 100 µM GSNO was examined through quantitative real-time RT-PCR. The data showed that under these conditions the *trHb* and *sdHb* expression underwent a fold increase of 1.7 ± 0.6 and 3.4 ± 1.3, respectively.

The growth of the Δ*trHb*, Δ*sdHb* and the Δ*trHb*Δ*sdHb* deletion mutants exposed to 100 µM GSNO was analysed. The Δ*sdHb* and double Δ*trHb*Δ*sdHb* mutant strains displayed higher sensitivity to nitrosative stress than the wild type strain. In the presence of GSNO, the growth of the Δ*trHb* mutant was not significantly different from that of the parental strain (Figure 6.2).

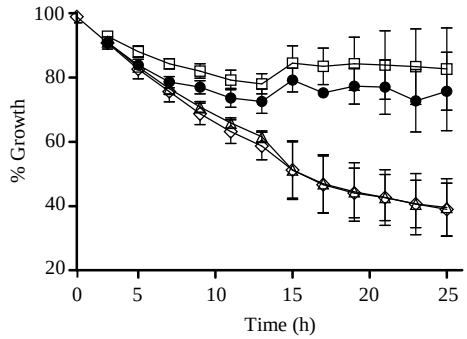


Figure 6.2 *H. pullorum* wild type and haemoglobin mutants under nitrosative stress. Growth of *H. pullorum* wild type (full circles), Δ*trHb* mutant (open squares), Δ*sdHb* mutant (open triangles) and Δ*trHb*Δ*sdHb* double mutant (open diamons) exposed to 100 µM GSNO. Percentage of growth represents the ratio of the OD_{600} of stress exposed versus untreated cells. Values are presented as means ± standard error of the mean of four independent experiments.

H. pullorum TrHb and SdHb protect *E. coli hmp* mutant from nitrosative stress

Complementation studies were conducted to test whether *H. pullorum* TrHb and SdHb proteins would be able to replace *E. coli* flavohemoglobin. Hence, *E. coli* Δ*hmp* mutant was transformed with pILL2157 plasmid expressing either *trHb* or *sdHb* gene. The *E. coli* Δ*hmp* strain transformed with the empty pILL2157 plasmid showed more susceptibility to 200 µM GSNO than the wild type strain transformed with the empty vector and the mutant strains expressing the TrHb and SdHb proteins (Figure 6.3). This shows that the expression of TrHb and SdHb from the inducible plasmid pILL2157-*trHb* and pILL2157-*sdHb* rescued the phenotype of *E. coli*, indicating that both *H. pullorum* TrHb and SdHb have the ability to alleviate the nitrosative stress generated by GSNO.

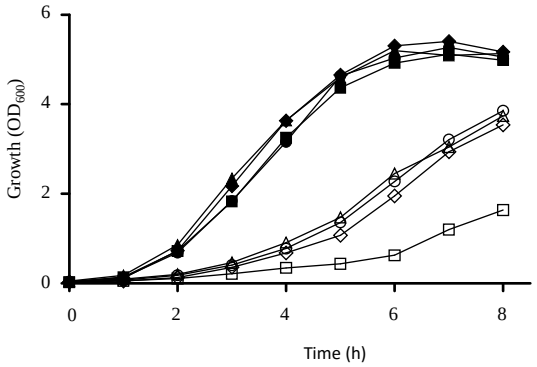


Figure 6.3 Complementation of the *E. coli* Δ*hmp* mutant with the *H. pullorum* TrHb and SdHb in the presence of nitrosative stress. Growth of *E. coli* wild type and Δ*hmp* mutant strains transformed with the empty vector pILL2157 (circles and squares respectively), and *E. coli* Δ*hmp* expressing the TrHb (triangles) or SdHb proteins (diamonds), in the absence of stress (full symbols) and in the presence of 200 µM GSNO (open symbols).

Survival of *H. pullorum* haemoglobins mutants in murine macrophages

The viability of the *H. pullorum* Δ*trHb* and Δ*sdHb* mutant strains were tested in murine J774A.1 macrophages. After 2 h of infection, no major differences in the survival of *H. pullorum* wild type and the mutants were observed. However, after 6 h of infection, while the Δ*trHb* mutant showed no major differences in macrophage survival in comparison with the wild type, the Δ*sdHb* and the double Δ*trHb*Δ*sdHb* mutants were less resistant to activated macrophages (Figure 6.4a). In addition, Δ*sdHb* and Δ*trHb*Δ*sdHb* mutants recovered their viability to the level of the Δ*trHb* mutant strain when in macrophages with the iNOS inhibited (Figure 6.4b). The viability of the double Δ*trHb*Δ*sdHb* mutant was slightly higher than that of Δ*sdHb*.

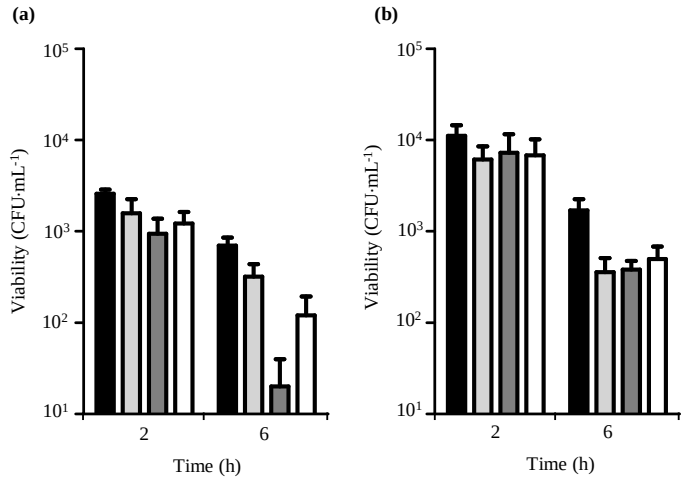


Figure 6.4 Survival of *H. pullorum* wild type and haemoglobin mutant strains upon macrophage infection. Activated (a) and iNOS inhibited (b) J774A.1 macrophages were infected with *H. pullorum* wild type (black bars), Δ*trHb* (light grey bars), Δ*sdHb* (dark grey bars) and Δ*trHb*Δ*sdHb* (white bars), at a MOI of 100. Viable counts were determined after 2 and 6 h of infection. Values are means ± standard error of the mean of at least two independent experiments analysed in duplicate.

Biochemical characterization of *H. pullorum* haemoglobins

The purified *H. pullorum* TrHb and SdHb have an apparent molecular mass of ~ 17 and 19 kDa respectively, which agrees with the molecular mass derived from the gene sequence plus the His-tag. TrHb and SdHb exhibit the typical features of haem containing proteins with Soret bands at 414 and 410 nm, respectively (Figure 6.5a and b). Furthermore, the redox spectra of the pyridine haemochrome of the *H. pullorum* TrHb and SdHb showed a band at 555 nm, with a ratio of contained 0.7 and 0.6 haem per protein, respectively, confirming the presence of haem *b* in both proteins.

The EPR spectra of the purified *H. pullorum* TrHb presents resonances characteristic of a high-spin haem, with g = 5.92. In agreement, a broad band in the UV-visible spectrum of the TrHb centred at approximately 630 nm was also observed (Figure 6.5a), which is characteristic of high spin haem proteins. In the

truncated haemoglobin group the haem spin state has been shown to vary amongst organisms (40), and in the *C. jejuni* Ctb it occurs as a mixture of low and high-spin haemic states (43).

The *H. pullorum* SdHb exhibits g-values of 3.23 and 2.97 that are typical of low spin haem containing proteins (Figure 6.5c). We speculate that tyrosine B10 residue (at position 29 in the amino acid sequence of *H. pullorum*, Figure 6.1) may act as the sixth haem ligand, as suggested for the group I truncated haemoglobin of the protozoan *Chlamydomonas eugametos* (71). The number of biochemically characterized bacterial single domain haemoglobins, is so far, limited to two *C. jejuni* and *Vitreoscilla* sp. strain C1 and in both cases, the haem is predominantly in the high spin state (72, 73). Thus, *H. pullorum* SdHb seems to have a different structural haem environment.

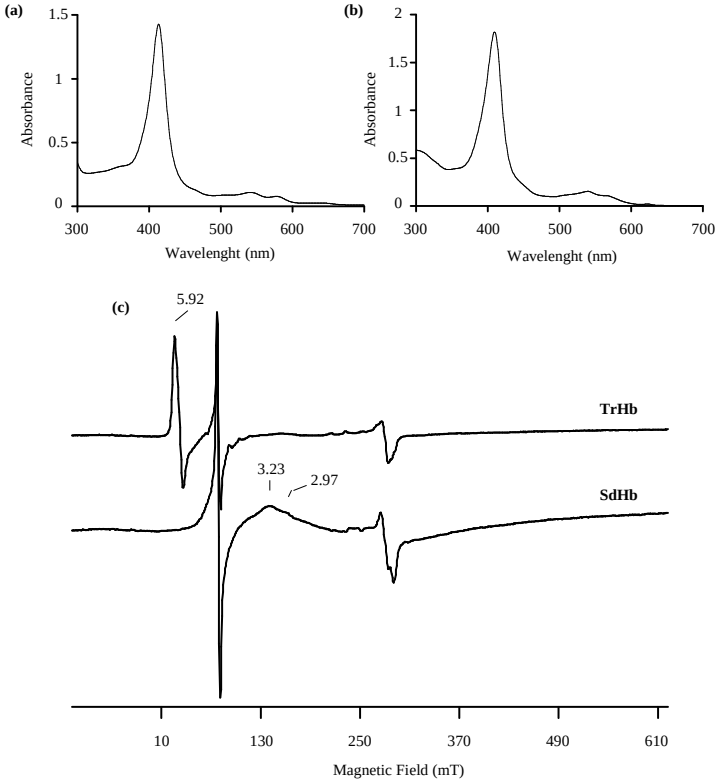


Figure 6.5 Spectroscopic studies of *H. pullorum* haemoglobins. UV-visible spectra of *H. pullorum* TrHb (a) and SdHb (b) and respective EPR spectra (c).

H. pullorum genome contains three peroxiredoxin-like genes

The analysis of the *H. pullorum* genome also revealed the presence of three putative peroxiredoxin-like homologs, namely HPMG_00817 (Prx1), HPMG_00739 (Prx2), and HPMG_ 00529 (Prx3)[2], which shown sequence similarity with the peroxiredoxins of the Bcp/PrxQ, AhpC/Prx1 and Tpx sub-families, respectively. The *H. pullorum* Prx1, Prx2 and Prx3 share, respectively, amino acid sequence identity and similarity with the Bcp (61% I/72% S), AhpC (69% I/60% S) and Tpx (83% I/72% S) proteins of *C. jejuni*, and with the Bcp (52% I/77% S), AhpC (46% I/66% S) and Tpx (87% I/66% S) peroxiredoxins of *H. pylori*.

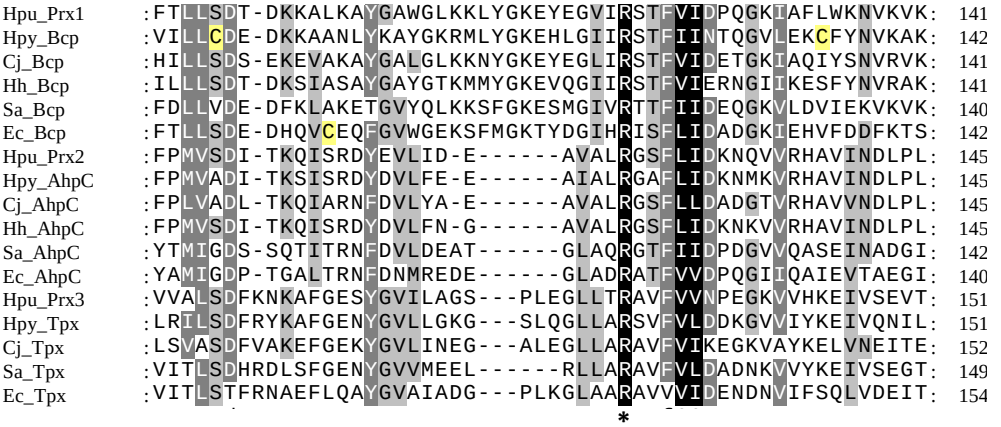
Peroxiredoxins are characterized by a highly conserved catalytic cysteine residue referred as the peroxidatic cysteine (C_P) in its catalytic centre (Figure 6.6). When present, a second conserved cysteine residue that forms a disulfide linkage with C_P during the catalytic cycle, termed resolving cysteine (C_R), is localized outside the catalytic centre (Figure 6.6). Moreover, besides the catalytic cysteines some peroxiredoxins contain other non-conserved cysteine residues that apparently are not involved in catalysis (*e.g.* S. *aureus* AhpC, *C. jejuni* and *E. coli* Tpx, Figure 6.6).

Comparison analysis of amino acid sequence of *H. pullorum* Prx1 shows the presence of a conserved cysteine at position 45, corresponding to a potential C_P and at position 50, corresponding to a potential C_R; *H. pullorum* Prx2 has two conserved cysteines, one at position 49 (a putative C_P) and another at 169 (a putative C_R), and an additional non-conserved cysteine at position 156. *H. pullorum* Prx3 has two conserved cysteines localised at position 58 (a putative C_P) and 92 (a putative C_R) (Figure 6.6). In addition to the C_P, peroxiredoxins contain the highly conserved proline, threonine and arginine residues in its catalytic centre (74) that we can see marked with one asterisk in Figure 6.6, and are present in the amino acid sequences of *H. pullorum* peroxiredoxins.

[2] *prx1*, *prx2* and *prx3* genes are also annotated as HPMG_RS04115, HPMG_RS03725 and HPMG_RS02600, respectively.


Hpu_Prx1 :----------MELQIGDKAPNFSLPNQDNAEISLQDFRGSWVVLYFYPK: 39
Hpy_Bcp :----------MEKLEVGQLAPDFRLKNSDGVEISLKDLLHKKVVLYFYPK: 40
Cj_Bcp :----------MSLNIGDKAPQFELLNQDGVKIALKDFIGKKVILYFYPK: 39
Hh_Bcp :----------MKLQKGDKAPQFRLKNADEIEISLQDLLTKRVVVYFYPK: 39
Sa_Bcp :----------MLQKGEQFPIFKLENQDGTVITNDTLKGKKAIIYFYPR: 38
Ec_Bcp :----------MNPLKAGDIAPKFSLPDQDGEQVNLTDFQGQRVLVYFYPK: 40
Hpu_Prx2 :----------MLVTKKAPNFKAPAVLADNQIVEDFELARNLGRNGAVVFFWPK: 43
Hpy_AhpC :----------MLVTKLAPDFKAPAVLGNNEVDEHFELSKNLGKNGAILFFWPK: 43
Cj_AhpC :----------MIVTKKALDFTAPAVLGNNEIVQDFNLYKNIGPKGAVVFFYPK: 43
Hh_AhpC :----------MLVTKPAPDFTAEAIKADGTFEDSFNLYKNIGKNGAVVFFWPK: 43
Sa_AhpC :----------MSLINKEILPFTAQAFDPKKDQFKEVTQEDLKGSWSVVCFYPA: 43
Ec_AhpC :----------MSLINTKIKPFKNQAFKNGEFIEITEKDTEGRWSVFFFYPA: 41
Hpu_Prx3 :---MVTFKGNAVSLKGKEINVGDSAPKVELIAGDLSAKSVGGASGKFQIINVVPS: 52
Hpy_Tpx :-MQKVTFKEETYQLEGKALKVGDKAPDVKLVNGDLQEVNLLKQGVRFQVVSALPS: 54
Cj_Tpx :--MSIVNFKGNPVKLKGNSVEVGADAPKVNLKAKDLSVIEIGAAGKTQIILSVPS: 53
Sa_Tpx :-MTEITFKGGPIHLKGQQINEGDFAPDFTVLDNDLNQVTLADYAGKKLLISVVPS: 54
Ec_Tpx :MSQTVHFQGNPVTVANSIPQAGSKAQTFTLVAKDLSDVTLGQFAGKRKVLNIFPS: 55
*


Hpu_Prx1 :DKTPGCTQEACDFRDNLANLSGLNAVVLGVSPDSVKTHQSFIDKE-------SLN: 87
Hpy_Bcp :DNTPGCTLEAKDFSALFSEFEKKNAVVVGISPDNAQSHQKFISQC-------SLN: 88
Cj_Bcp :DNTPGCTTEACDFSANYDKFGGKNAVIIGISPDSVASHEKFISKF-------DLK: 87
Hh_Bcp :DNTPGCTIEAEEFSTLLEKFEAKDTIIVGISPDSPKCHQNFINKK-------ALK: 87
Sa_Bcp :DNTPTCTTEACDFRDNLEMFNDLDVAVYGISSDSKKKHQNFIEKH-------GLN: 86
Ec_Bcp :AMTPGCTVQACGLRDNMDELKKAGVDVLGISTDKPEKLSRFAEKE-------LLN: 88
Hpu_Prx2 :DFTFVCPSEIIAMDHRVKAFAEKGFNVIGVSIDSDVVHFAWKNTPVNQGGIGNVQ: 98
Hpy_AhpC :DFTFVCPTEIIAFDKRVKDFQEKGFNVIGVSIDSEQVHFAWKNTPVEKGGIGQVT: 98
Cj_AhpC :DFTFVCPSEIIAFDKRYQEFKNRGIEVIGISGDNEFSHFAWKNTPVNQGGIGQVK: 98
Hh_AhpC :DFTFVCPSEIIAFDKRVKDFEARGVKVIGVSIDSKEVHFAWRNVPVNQGGIGAVT: 98
Sa_AhpC :DFSFVCPTELEDLQNQYEELQKLGVNVFSVSTDTHFVHKAWHDHS---DAISKIT: 95
Ec_AhpC :DFTFVCPTELGDVADHYEELQKLGVDVYAVSTDTHFTHKAWHSSS---ETIAKIK: 93
Hpu_Prx3 :LDTGVCATQTRKFNEKAASLS--NAEVFVVSLDLPFAQGRFCSIE------GIQN: 99
Hpy_Tpx :LTGSVCQ--AKHFNEQTGKLP--SVSFSVISMDLPFSQGQICGAE------GIKD: 99
Cj_Tpx :LDTPVCATEAREFNKKVASYN--GAEVIVVSMDLPFAMGRFCSTE------GIEN: 100
Sa_Tpx :IDTGVCDQQTRKFNSDASKE---EGIVLTISADLPFAQKRWCASA------GLDN: 100
Ec_Tpx :IDTGVCAASVRKFNQLATEID--NTVVLCISADLPFAQSRFCGAE------GLNN: 102
*


Hpu_Prx1 :FTLLSDT-DKKALKAYGAWGLKKLYGKEYEGVIRSTFVIDPQGKIAFLWKNVKVK: 141
Hpy_Bcp :VILLCDE-DKKAANLYKAYGKRMLYGKEHLGIIRSTFIINTQGVLEKCFYNVKAK: 142
Cj_Bcp :HILLSDS-EKEVAKAYGALGLKKNYGKEYEGLIRSTFVIDETGKIAQIYSNVRVK: 141
Hh_Bcp :ILLLSDT-DKSIASAYGAYGTKMMYGKEVQGIIRSTFVIERNGIIKESFYNVRAK: 141
Sa_Bcp :FDLLVDE-DFKLAKETGVYQLKKSFGKESMGIVRTTFIIDEQGKVLDVIEKVKVK: 140
Ec_Bcp :FTLLSDE-DHQVCEQFGVWGEKSFMGKTYDGIHRISFLIDADGKIEHVFDDFKTS: 142
Hpu_Prx2 :FPMVSDI-TKQISRDYEVLID-E------AVALRGSFLIDKNQVVRHAVINDLPL: 145
Hpy_AhpC :FPMVADI-TKSISRDYDVLFE-E------AIALRGAFLIDKNMKVRHAVINDLPL: 145
Cj_AhpC :FPLVADL-TKQIARNFDVLYA-E------AVALRGSFLLDADGTVRHAVVNDLPL: 145
Hh_AhpC :FPMVSDI-TKQISRDYDVLFN-G------AVALRGSFLIDKNKVVRHAVINDLPL: 145
Sa_AhpC :YTMIGDS-SQTITRNFDVLDEAT------GLAQRGTFIIDPDGVVQASEINADGI: 142
Ec_AhpC :YAMIGDP-TGALTRNFDNMREDE------GLADRATFVVDPQGIIQAIEVTAEGI: 140
Hpu_Prx3 :VVALSDFKNKAFGESYGVILAGS---PLEGLLTRAVFVVNPEGKVVHKEIVSEVT: 151
Hpy_Tpx :LRILSDFRYKAFGENYGVLLGKG---SLQGLLARSVFVLDDKGVVIYKEIVQNIL: 151
Cj_Tpx :LSVASDFVAKEFGEKYGVLINEG---ALEGLLARAVFVIKEGKVAYKELVNEITE: 152
Sa_Tpx :VITLSDHRDLSFGENYGVVMEEL------RLLARAVFVLDADNKVVYKEIVSEGT: 149
Ec_Tpx :VITLSTFRNAEFLQAYGVAIADG---PLKGLAARAVVVIDENDNVIFSQLVDEIT: 154
*

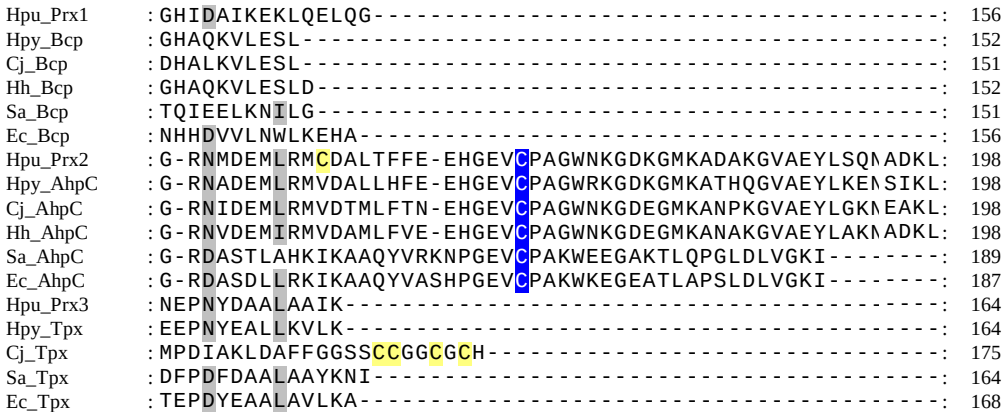


Figure 6.6 Amino acid sequence comparison of *H. pullorum* HPMG_00817, HPMG_00739, and HPMG_ 00529 gene products with the best studied bacterial peroxiredoxins. Protein sequences: *H. pullorum* (Hpu_Prx1), *H. pylori* (Hpy_Bcp), *C. jejuni* (Cj_Bcp), *H. hepaticus* (Hh_Bcp), *S. aureus* (Sa_Bcp) and *E. coli* (Ec_Bcp) peroxiredoxins belonging to Bcp/PrxQ subfamily; *H. pullorum* (Hpu_Prx2), *H. pylori* (Hpy_AhpC), *C. jejuni* (Cj_AhpC), *H. hepaticus* (Hh_AhpC), *S. aureus* (Sa_AhpC), and *E. coli* (Ec_AhpC) belonging to AhpC/Pxr1 subfamily; and *H. pullorum* (Hpu_Prx3), *H. pylori* (Hpy_Tpx), *C. jejuni* (Cj_Tpx), *S. aureus* (Sa_Tpx) and *E. coli* (Ec_Tpx) peroxiredoxins belonging to Tpx subfamily. The potential peroxidatic cysteine residue is shaded in red, the potential resolving cysteine in blue and the non-conserved cysteines in yellow. The conserved proline and threonine present at the catalytic site and the distal conserved arginine are marked with an asterisk. Alignments were done using the Clustal X algorithm.

Expression of *H. pullorum* peroxiredoxins in stressed cells

Since peroxiredoxins are usually involved in oxidative stress defence, we also studied the expression pattern of *H. pullorum* peroxiredoxin genes and the phenotype of the mutant strains towards hydrogen peroxide. The expression of the peroxiredoxin genes in *H. pullorum* treated with 50 µM of hydrogen peroxide, for 30 min, and with 50 µM peroxynitrite, for 15 min, was quantified by quantitative real-time RT-PCR. Data showed that the expression of the *prx2* gene was increased

in the presence of peroxynitrite and the expression of *prx3* was augmented in cells exposed to hydrogen peroxide. The *prx1* gene did not suffer a significant increase in its expression in response to the same stress generators (Figure 6.7).

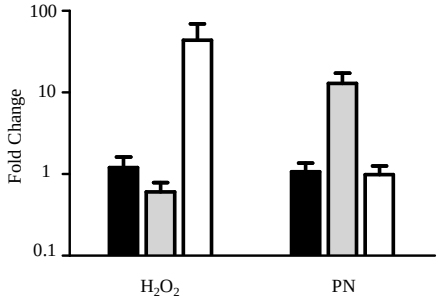


Figure 6.7 Effect of the oxidative and nitrosative stress on the transcription of *H. pullorum* peroxiredoxin genes. Fold variations of the expression of the genes *prx1* (black bars), *prx2* (grey bars), and *prx3* (white bars), upon exposure of the *H. pullorum* wild type strain to 50 µM hydrogen peroxide (H_2O_2) or 50 µM peroxynitrite (PN). Fold change values represent the ratio of the expression level of treated culture to that of untreated, and is adjusted to the constitutive *gyrA* gene expression. At least two biological samples were analysed in duplicate.

Phenotypical analysis of *H. pullorum* peroxiredoxin mutants

Single Δ*prx1*, Δ*prx2* and double Δ*prx1*Δ*prx2 H. pullorum* deletion mutants were constructed. However, in spite of several attempts, the Δ*prx3* deletion was not successful that suggests the *prx3* gene is essential for *H. pullorum*.

Even in the absence of the stress, the Δ*prx2* and Δ*prx1*Δ*pxr2* mutants showed a significantly decrease of the growth rate and viability (Figure 6.8). Therefore, the single Δ*prx2* and double Δ*prx1*Δ*pxr2* mutants were excluded from the subsequent studies. We tested the growth of these strains in two different types of liquid medium, because, the subsequent susceptibility assays with hydrogen peroxide would be performed in BHI, the usual medium for *Helicobacter* growth,

while the assays with peroxynitrite would be performed with BB, as peroxynitrite seems to decompose quickly in contact with BHI.

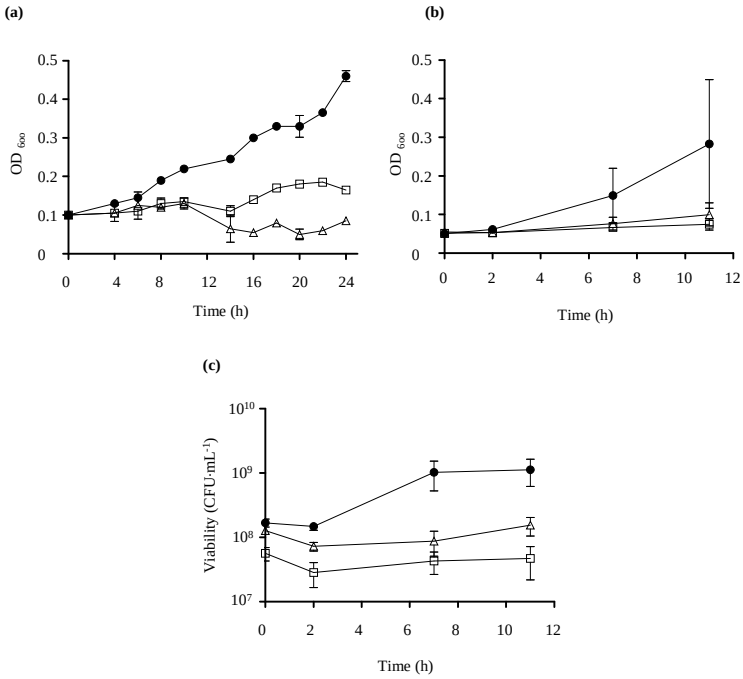


Figure 6.8 Behaviour of *H. pullorum* Δ*prx2* and Δ*prx1*Δ*prx2* mutants under normal growth conditions. Growth (a, b) and viability (c) of *H. pullorum* wild type (full circles), Δ*prx2* mutant (open triangles) and double Δ*prx1*Δ*prx2* mutant (open squares) in BHI-βCD (a) and in BB complemented with 2.5% FCS (b, c). Values represent the mean ± standard error of the mean of two independent experiments.

Growth of the *H. pullorum* Δ*prx1* mutant was analysed under stress conditions generated by 1 mM and 5 mM hydrogen peroxide, and 50 µM peroxynitrite. Its viability was also analysed upon infection of J774A.1 macrophages.

H. pullorum Δ*prx1* mutant showed no increased sensitivity to hydrogen peroxide (Figure 6.9a and b) and peroxynitrite (Figure 6.9c), which suggests that Prx1 does not contribute to the stress protection of *H. pullorum*. Also, no significant

differences were seen in the survival of the Δ*prx1* mutant comparatively to the wild type during macrophage infection (Figure 6.10).

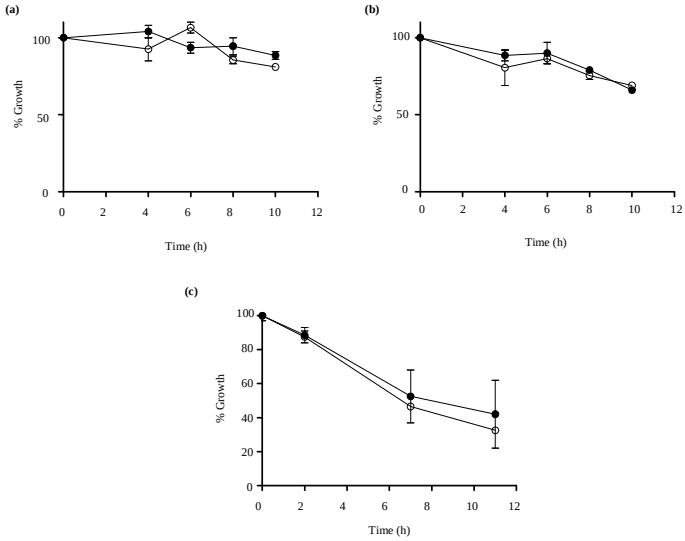


Figure 6.9 Effect of oxidative and nitrosative stress on *H. pullorum* wild type and Δ*prx1* mutant. Growth of *H. pullorum* wild type (full circles) and Δ*prx1* mutant (open circles) in BHI-βCD medium with 1 mM (a) and 5 mM (b) hydrogen peroxide, and in BB complemented with 2.5% FCS and 50 µM peroxynitrite (c). Values represent the mean ± standard error of the mean of two independent experiments.

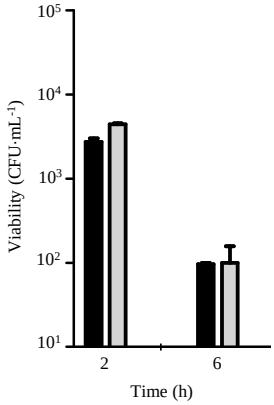


Figure 6.10 Survival of *H. pullorum* wild type and the *prx1* mutant upon macrophage infection. Activated J774A.1 macrophages were infected with *H. pullorum* wild type (black bars) and Δ*prx1* mutant (light grey bars), at a MOI of 100. Viable counts were determined after 2 and 6 h of infection. Values are means ± standard error of the mean of one experiment analysed in triplicate.

Prx1, Prx2 and Prx3 have peroxynitrite reductase activity

As observed in SDS/PAGE gel, the purified *H. pullorum* Prx1, Prx2 and Prx3 have a molecular mass of approximately 20, 24 and 19 kDa, respectively, which agrees with the molecular mass derived from the gene sequence plus the His-tag. The UV–Visible spectra of the *H. pullorum* peroxiredoxins indicate that these proteins have no metal co-factors. Peroxynitrite proved to be promptly degraded by the reduced proteins Prx1, Prx2 and Prx3. A representative data set collected, at 5 °C, is shown in Figure 6.11. From the kinetic traces acquired at 310 nm, it can be clearly appreciated that peroxynitrite, when mixed with the buffer alone (100 mM phosphate buffer pH = 7.0 plus 0.2 mM DTPA), is stable over the first 100 ms (dashed lines). A significantly faster decomposition is instead observed when peroxynitrite is mixed with any of the three proteins in the reduced state (solid lines). At 5 °C, the reaction with Prx1 or Prx2 was sufficiently slow to be time-resolved by stopped-flow technique (Figures 6.11a and 6.11b). In contrast, despite the low temperature, the reaction with Prx3 was much faster and a significant

fraction of the reaction took place over the first milliseconds, thus overlapping with a small artefactual signal invariantly observed immediately after mixing (Figure 6.11c). Therefore, whereas the initial rates could be reliably measured for the reaction of peroxynitrite with Prx1 and Prx2, such analysis could not be performed in the case of Prx3, the reaction being too fast. As a control, we confirmed that no reaction is observed over the same time scale (100 ms), if the *H. pullorum* proteins are pre-oxidized with an excess of hydrogen peroxide (~ 300 µM) prior to mixing with peroxynitrite (not shown).

In agreement with previous reports (75), in the case of Prx1 and Prx2 the initial rate of peroxynitrite decomposition was found to be proportional to the protein concentration, the reaction with Prx1 being slightly faster than with Prx2 (~ 5 x 10^5 $M^{-1} \cdot s^{-1}$ vs ~ 2 x 10^5 $M^{-1} \cdot s^{-1}$) (Figure 6.12). Peroxynitrite decomposition by reduced Prx3 was much faster than observed with Prx1 and Prx2, the reaction time courses being compatible with a second-order rate constant k above 10^6 $M^{-1} \cdot s^{-1}$. The k values estimated for the *H. pullorum* Prx1 and Prx2, are similar to those measured at the same temperature for the *Giardia* peroxiredoxins (75). The values fall within the range reported for peroxiredoxins from different microbial sources (69), taking into account that the published values were obtained at higher temperature (25 °C or 37 °C).

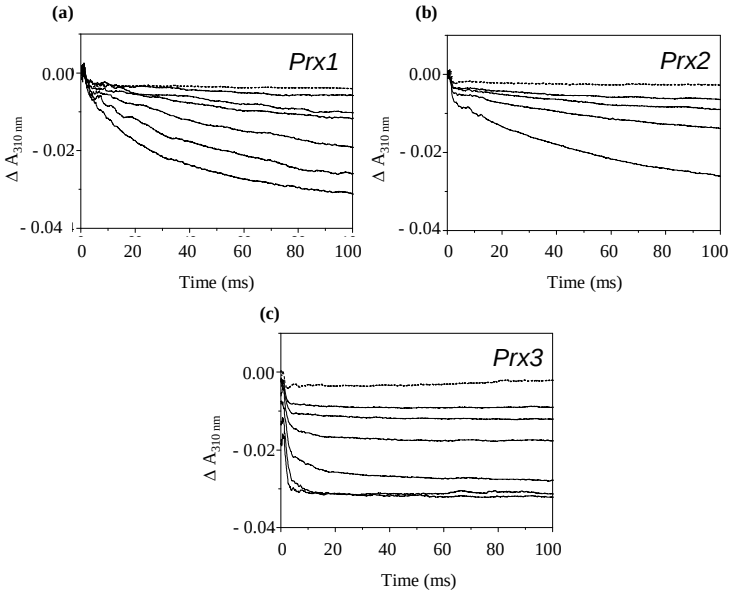


Figure 6.11: Reaction of reduced *H. pullorum* peroxiredoxins with peroxynitrite. Absorption changes measured at 310 nm after anaerobically mixing peroxynitrite ($ONOO^-$) with Prx1 (a), Prx2 (b) or Prx3 (c) at increasing concentrations. Temperature = 5 °C. Concentrations after mixing: (A) [Prx1] = 0 - 2.0 - 4.0 - 8.1 - 16.2 - 32.5 - 65.0 µM, [$ONOO^-$] = 19 µM; (B) [Prx2] = 0 - 10.1 - 21.2 - 42.5 - 85.0 µM, [$ONOO^-$] = 24 µM. (C) [Prx3] = 0 - 2.5 - 5.0 - 10.1 - 21.2 - 42.5 - 85.0 µM, [$ONOO^-$] = 20 µM.

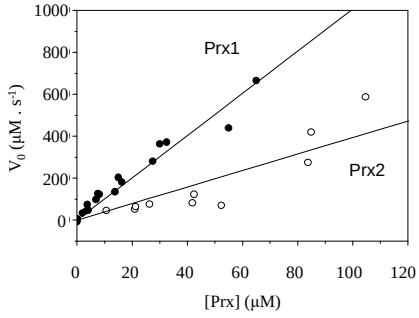


Figure 6.12 Initial rate of peroxynitrite decomposition by *H. pullorum* Prx1 and Prx2. The initial rate of peroxynitrite consumption measured, at 5 °C, at increasing

concentrations of Prx1 (closed circles) and Prx2 (open circles). From linear regression analysis, the following second-order rate constants were estimated: $k \sim 5 \times 10^5\ M^{-1} \cdot s^{-1}$ (Prx1) and $k \sim 2 \times 10^5\ M^{-1} \cdot s^{-1}$ (Prx2).

6.4 Discussion

We showed that *H. pullorum* contains two haemoglobins that are highly similar to the single domain haemoglobin (Cgb) and to the group III truncated haemoglobins of *C. jejuni* (Ctb). *H. pullorum* haemoglobins were induced in response to GSNO, as *C. jejuni cgb* and *ctb*, which were also shown to be up-regulated in response to several nitrosative stress generators, including GSNO through the NO-responsive regulator NssR (28, 39, 45). Another indication for the role of *H. pullorum* haemoglobins in NO protection was the complementation assays showing that the GSNO sensitive phenotype of the *E. coli* mutant lacking the NO-detoxifying flavohemoglobin (Hmp) was relieved upon expression of *H. pullorum* SdHb and TrHb. According to our results, the single domain haemoglobins Cgb of *C. jejuni* (26) and Vhb of *Vitreoscilla* fused with a flavoreductase domain from *R. eutropha* (30), as well as the group I HbN of *M. tuberculosis* (24) and group II HbO of *P. haloplanktis* (36) were also shown to restore the growth defect of an *E. coli* Δ*hmp* mutant exposed to nitrosative stress. *M. tuberculosis* HbN overexpression also provided substantial protection for the *Salmonella enterica Typhimurium hmp* mutant growing in the presence of nitrogen species (31). Furthermore, the expression of the group III truncated haemoglobin Ctb of *C. jejuni* in the *E. coli* Δ*hmp* mutant protected aerobic respiration from NO inhibition compared to cells carrying an empty vector (26) although it did not complement the growth defect of the *E. coli* Δ*hmp* (38).

The *H. pullorum* Δ*sdHb* mutant exhibited a higher sensitivity towards nitrosative stress generated by GSNO, as observed for the *C. jejuni ctb* mutant (26, 28). Furthermore, in our study, *H. pullorum* SdHb improved the ability of *H. pullorum* to survive to the nitrosative stress generated by macrophages. Therefore, as in the case of *C. jejuni* Cgb, our results indicate a role of *H. pullorum* SdHb in nitrosative stress protection.

In spite of upregulated in the presence of nitrosative stress, and have restored the phenotype of an *E. coli* Δ*hmp*, the *H. pullorum* Δ*trHb* mutant was not sensitive to the nitrosative stress generated by GSNO, which suggests a predominant role of *H. pullorum* SdHb over TrHb in nitrosative stress defence (76). This phenotype pattern observed for *H. pullorum* TrHb was similar to the one observed for *C. jejuni* Ctb, with the exception that *H. pullorum* TrHb complemented the growth defect of *E. coli* Δ*hmp* in the presence of GSNO and *C. jejuni* Ctb did not. Furthermore, TrHb did not improve the *H. pullorum* survival upon macrophage infection. This result differs from that of *M. tuberculosis* HbN in macrophage survival, as the overexpression of HbN was shown to improve the survival of *M. tuberculosis* within THP-1 and mouse peritoneal macrophages (77), and the survival of the heterologous *hmp* deficient *Salmonella* within mouse peritoneal macrophages (31). This difference may be explained by the different number and type of the haemoglobin genes present in their respective genomes that may lead to the same enzymatic functions occurring in different haemoglobin classes. Since *M. tuberculosis* has two truncated haemoglobins, and *H. pullorum* has one truncated and one single domain haemoglobin, the SdHb of the latter may exert a similar function as the *Mycobacterium* HbN truncated haemoglobin.

Interestingly, the double Δ*sdHb*/Δ*trHb H. pullorum* mutant was not as sensitive as the Δ*sdHb* upon macrophage infection, possible due to the up-regulation of other *H. pullorum* defences in consequence of the double mutation. These observations are in agreement with the *C. jejuni* haemoglobin studies, in which the double mutant lacking both *cgb* and *ctb* genes was more resistant to GSNO than the single *cgb* mutant, but slightly sensitive than the wild type and *ctb* mutant strains (26).

H. pullorum prx1, the homolog of bacterial Bcps, was not induced and did not conferred protection from hydrogen peroxide. Contrary to our results, *Burkholderia cenocepacia* (78), *E. coli* (52) and *P. gingivalis* (79) *bcp* mutants were susceptible to hydrogen peroxide, and the *bcp* deficient *H. pylori* was shown a weaker sensitivity to hydrogen peroxide (47). Furthermore, in *E. coli* and *H. pylori*, Bcp was shown to detoxify hydrogen peroxide, but with a lower efficiency than bacterial AhpC and Tpx proteins, and exhibited a preference for organic peroxides (52, 80, 81). Although *prx1* was not induced and did not conferred protection from

peroxynitrite, the Prx1 protein was endowed with peroxynitrite activity, which points for a role of this protein in nitrosative stress protection. Since bacteria typically possess multiple scavenging systems for the same toxic substrate (82), the lack of phenotype observed for the *H. pullorum* Δ*prx1* mutant could be explained by the presence of another enzymes in *H. pullorum* that detoxify hydrogen peroxide and peroxynitrite, and may compensate the Prx1 absence. Also, the *C. jejuni* single *bcp* was not more susceptible than the wild type strain to hydrogen peroxide and nitrosative stress agents however, a double mutation in the *bcp* and *tpx* genes rendered the strain hypersensitive (48). Finally, as observed in our study for *H. pullorum* Prx1, *Burkholderia* Bcp was not required for macrophage survival (78).

The *H. pullorum* Δ*prx2* and Δ*prx1*/Δ*pxr2* mutants showed a growth defect even under normal growth conditions. Previous work also reported that the *ahpC* of *H. pylori*, a homolog of *H. pullorum prx2*, is a critical factor for *H. pylori* viability as the *H. pylori ahpC* mutants were only obtained under low oxygen conditions and were highly susceptible to the normal microaerobic growth conditions of *H. pylori* (59, 83). *H. pullorum prx2* was up-regulated in response to peroxynitrite and showed activity towards peroxynitrite. The *S. typhimurium*, *M. tuberculosis*, and *H. pylori* AhpC were shown to detoxify peroxynitrite with similar second order rate constants as *H. pullorum* Prx2 (53).

The construction of the *prx3* deletion strain was hindered, which suggests that this peroxiredoxin may be essential in *H. pullorum*. Interestingly, Prx3 was shown to be upregulated in response to hydrogen peroxide, suggesting a role of this protein in protecting *H. pullorum* from oxidative stress. Furthermore, Prx3 exhibited the highest peroxynitrite activity among the three *H. pullorum* peroxiredoxins, suggesting an important role in peroxynitrite detoxification. In similarity with *M. tuberculosis* Tpx, Prx3 was shown to reduce peroxynitrite with an activity of approximately one log higher than the ones reported for the bacterial AhpC enzymes (53, 54).

In conclusion, this is the first study focusing on the defence mechanisms of the potential human pathogen *H. pullorum* against the antimicrobials produced by the innate immunity. We reported that a single domain globin has a main role in the

protection of *H. pullorum* and that peroxiredoxins are also involved in nitrosative stress protection of *H. pullorum*.

6.5 Acknowledgments

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Discussion

Chapter VII

General discussion

7.1 *H. pullorum* interaction with the host

H. pullorum has been considered an emergent foodborne pathogen as it was recently isolated from fresh poultry meat and has been associated with several gastrointestinal and hepatic human disorders (1). In spite of its pathogenic potential, the infection process and the host immune response to this bacterium are poorly studied. Our work provides new data regarding the interaction of *H. pullorum* with the host innate immune cells.

We have demonstrated that *H. pullorum* is phagocytised by mammalian macrophages. Similar results were observed for *H. pylori* that even though usually classified as an extracellular pathogen, can penetrate the epithelial barrier and be phagocytized.

Our data further revealed that *H. pullorum* increases the production of NO by host macrophages. Also, *H. pylori* and *H. hepaticus* (2) were previously shown to induce the transcription of the enzyme iNOS. Furthermore, *H. pullorum* was shown to be susceptible to macrophage killing in a NO-dependent manner. Similarly, *H. pylori* was shown to be susceptible to the NO generated by activated macrophages. However, studies reported that when in contact with non-activated macrophages, some *H. pylori* strains can downregulate NO production, inhibit its internalisation by phagocytes or impair the maturation of phagosomes upon phagocytosis, thus being able to survive the host immune response (see Chapter 2). Our study suggests that RNS contribute for *H. pullorum* elimination. We also observed a correlation between the levels of NO produced by macrophages and the levels of *H. pullorum* phagocytised. Similar behaviour was so far only observed for *P. gingivalis* (3) and *S. aureus* (4), to which a higher production of NO correlated with higher bacterial phagocytosis.

This work showed that *H. pullorum* triggers the expression of the inflammatory genes TNF-α, IL-1β, IL-6 and MIP-2 (the murine homolog of IL-8) upon contact with murine macrophages. Other *Helicobacter* species were reported to promote the transcription of inflammatory cytokines in host cells and to induce a strong local inflammatory response, which is correlated with the promotion of cellular lesions that may lead to carcinogenesis. In particular, *H. pylori* and *H.*

hepaticus induce the expression of TNF-α, IL-1β, IL-6 and IL-8 by host cells (2, 5, 6).

The induction of cytokine transcription promoted by *H. pullorum*, is consistent with their ability to increase NO production in macrophages, as the observed induced cytokines have been associated with iNOS activation (7). Moreover, the data are in agreement with previous studies showing that *H. pullorum* induced IL-8 in human gastric and intestinal epithelial cell lines (8, 9). The induction of IL-8 secretion in epithelial cells was shown to be mediated by *H. pullorum* cytolethal distending toxin (CDT) through the NF-κβ pathway, but a CDT-independent IL-8 induction was also reported in the same study. As *H. pullorum* LPS has the highest biological activity within the *Helicobacter* genus, and promoted NF-κβ activation, those authors suggested that in addition to CDT, the IL-8 expression could also be promoted by the LPS of *H. pullorum* (9, 10). Therefore, the activation of macrophages by *H. pullorum* may involve NF-kB pathway activation through *H. pullorum* CDT activity and by interaction of LPS with the host cells. In contrast to the highly reactive LPS of *H. pullorum* and other Gram-negative bacteria such as *E. coli* and *S. typhimurium* (11), *H. pylori* LPS has a remarkably low reactivity, which seem to be used as a strategy to avoid macrophage activation (see Chapter 2).

The effect of NO in *H. pullorum* viability and cell morphology was analysed. *H. pullorum* was shown to be susceptible to the *in vitro* nitrosative stress generated by several nitrosative stress agents in a concentration-dependent manner, as also observed for other human pathogens such as *H. pylori* (12). *H. pullorum* susceptibility to NO was also dependent on the bacterial growth phase at which the stress was applied, with *H. pullorum* cells being substantially more susceptible at the earlier exponential than at the exponential and stationary phases. *H. pylori* also responds differently to environmental changes on a growth phase dependent manner. For example, while *H. pylori* exponential growing cells were rapidly killed by iron depletion, the stationary-phase cells showed a remarkable ability to survive. Moreover, under iron starvation *H. pylori* exhibited a gene transcription pattern that varies with the growth phase (13).

Exposure of *H. pullorum* to nitrosative stress generated by spermine-NONOate (0-0.1 mM for 24 h), did not result in the formation of coccoid cells. Several studies reported that some *Helicobacter* species change their morphology from a bacillary to a coccoid form, in response stresses, such as acid pH, temperature, nutritional starvation, and oxidative and nitrosative stress. *H. pylori* NCTC 11637 is rapidly converted to a coccoid morphology under low concentrations of peroxynitrite (14). Also, Cole and co-workers reported that GSNO causes the conversion of *H. pylori* SD14 bacilli to a coccoid morphology at a concentration of 0.1 mM for 24 h, or after 4 h of incubation at a concentration of 3 mM (15). However, *for H. pylori* 26695 and B128 strains, Saraiva and co-workers reported that GSNO (200 µM) did not induced morphological changes (16). Therefore, the morphological conversion of *H. pylori* in response to nitrosative stress seems to be dependent on the strain.

Although the formation of *H. pullorum* coccoid cells was not observed, exposure to nitrosative stress induced a decrease in the average length of the cells and the formation of some "U", "V" and "S" cell shapes, which may signify the presence of some *H. pullorum* cells in an earlier stage of cocci conversion. In other studies, "U", "V" and "S" *H. pylori* cell shapes were described as intermediate forms that occur before the complete cellular conversion from bacilli to cocci (17, 18).

Previous work showed that *H. pullorum* i) is able to invade human intestinal cells (19), ii) promotes cellular toxicity through the expression of CDT (20), and iii) induce an inflammatory response in epithelial human cell lines. This work further informed that *H. pullorum* triggers inflammation in the host and that is susceptibility to high levels of NO.

7.2 Defence mechanisms of *Helicobacter* species against nitrosative stress

In spite of the data demonstrating the capacity of *H. pullorum* to activate the innate immunity and its susceptibility to nitrosative stress ((8, 9), Chapter VI), until now, no mechanisms of protection against nitrosative stress were studied in *H. pullorum*. The genome of *H. pullorum* has two haemoglobin-like genes (HPMG_00954 and HPMG_00979), which products belong to the single domain

haemoglobin and to the group III truncated haemoglobin families, in contrast of what is observed for *H. pylori*. These proteins present a high degree of sequence similarity to the *C. jejuni* single domain (Cgb) and truncated (Ctb) haemoglobins. The single domain haemoglobin of *H. pullorum* (SdHb) was shown to be induced by GSNO, restore the growth of an *E. coli* mutant lacking the NO-detoxifying flavohaemoglobin (Hmp), and protect bacterial growth from GSNO (Chapter VI). A similar phenotype was observed for the Cgb of *C. jejuni* (see Chapter III, section 3.4.2).

The similar functions observed for *H. pullorum* SdHb and *C. jejuni* Cgb are in accordance with their high degree of amino acid sequence similarity. Although also sharing a high degree of similarity with *C. jejuni* Cgb and *H. pullorum* SdHb, the single domain haemoglobin of *Vitreoscilla* (Vhb) seems to have a major role in oxygen transport and oxidative stress protection instead of being involved in nitrosative stress defence (Chapter III, section 3.4).

In agreement with their high sequence similarity, both *H. pullorum* and *C. jejuni* truncated haemoglobins have a similar phenotype in the presence of NO. As for *C. jejuni* Ctb, the gene encoding the *H. pullorum* truncated haemoglobin was up-regulated by GSNO, and upon expression restored the GSNO sensitive phenotype of an *E. coli hmp* mutant strain. Nevertheless, the mutant strains were not sensitive to GSNO. Less conflicting results were obtained for the group I *M. tuberculosis* HbN, and the group II *M. leprae* HbO and *P. haloplanktis* Ph-2/2HbO truncated haemoglobins, which were demonstrated to detoxify NO, or peroxynitrite in the case of Ph-2/2HbO, and to protect against nitrosative stress (see Chapter III, section 3.4). Our *in vivo* results corroborate the *in vitro* phenotype of the *H. pullorum* haemoglobins mutants demonstrated in the GSNO growth susceptibility assays, as the *H. pullorum* SdHb but not the TrHb, contributes to the ability of *H. pullorum* to survive to macrophage killing. Altogether, these observations suggest a primary role of the single domain haemoglobin relatively to the truncated haemoglobin in the defence of *H. pullorum* and *C. jejuni* against nitrosative stress.

H. pullorum has three peroxiredoxins-like sequences belonging to the Bcp/PrxQ (HPMG_00817), AhpC/Prx1 (HPMG_00739) and Tpx (HPMG_ 00529) families,

as is observed for *H. pylori* and *C. jejuni*, herein named *H. pullorum* Prx1, Prx2 and Prx3, respectively. *H. pullorum* Prx1 has peroxynitrite activity, which value is within the range of values reported for other bacterial peroxiredoxins (Table 7.1). To date, no other bacterial peroxiredoxin belonging to the same family was reported to detoxify peroxynitrite. A redundant role of peroxiredoxins in stress defence due to its overlapping substrate specificities was previously reported in other organisms. Since *H. pullorum* has three peroxiredoxins and all three showed activity towards peroxynitrite, it is possible that the activity of Prx2 and Prx3 may compensate for the loss of *prx1*, thus explaining the lack of *H. pullorum prx1* phenotype towards peroxynitrite. It was previously described that *E. coli* and *H. pylori* Bcp, the protein homolog of *H. pullorum* Prx1, detoxifies hydrogen peroxide, and that *E. coli bcp* protected against hydrogen peroxide. Contrary to *E. coli bcp*, *H. pullorum prx1* was not induced by hydrogen peroxide, and did not confer growth protection from hydrogen peroxide. It is possible that the phenotype is masked by the activity of the two other peroxiredoxins, or even of enzymes, such as catalase that may compensate the lack of *prx1*.

Table 7.1 Reported second order rate constants for peroxynitrite reduction by peroxiredoxins

Peroxiredoxin	Peroxiredoxin subfamily	Rate constant ($M^{-1} \cdot s^{-1}$)	Conditions	References
Helicobacter pylori AhpC	Prx1/AhpC	1.2×10^6	pH 6.8; 25 ºC	(21)
Helicobacter pullorum Prx2	Prx1/AhpC	2.0×10^5	pH 7.0; 5 ºC	This study
Mycobacterium tuberculosis AhpC	Prx1/AhpC	1.3×10^6	pH 6.8; 25 ºC	(21)
Salmonella typhimurium AhpC	Prx1/AhpC	1.5×10^6	pH 6.8; 25 ºC	(21)
Human Prx2	Prx1/AhpC	1.4×10^7	pH 7.4; 25 ºC	(22)
Plasmodium falciparum TPx1	Prx1/AhpC	1.0×10^6	pH 7.4; 37 ºC	(23)
Sacharomyces cerevisiae Tsa1	Prx1/AhpC	7.4×10^5	pH 7.4; 25 ºC	(24)
Sacharomyces cerevisiae Tsa2	Prx1/AhpC	5.1×10^5	pH 7.4; 25 ºC	(24)
Trypanosoma brucei TXNPx	Prx1/AhpC	9.0×10^5	pH 7.4; 37 ºC	(25)
Trypanosoma cruzi TXNPx	Prx1/AhpC	7.2×10^5	pH 7.4; 37 ºC	(25)
Giardia intestinalis Prx1a	Prx1/AhpC	4.0×10^5	pH 7.0; 4 ºC	(26)
Giardia intestinalis Prx1b	Prx1/AhpC	2.0×10^5	pH 7.0; 4 ºC	(26)
Helicobacter pullorum Prx1	Bcp/PrxQ	5.0×10^5	pH 7.0; 5 ºC	This study
Xylella fastidiosa PrxQ	Bcp/PrxQ	1.0×10^6	pH 7.4; 37 ºC	(27)
Helicobacter pullorum Prx3	TPx	$>1.0 \times 10^6$	pH 7.0; 5 ºC	This study
Mycobacterium tuberculosis TPx	TPx	1.5×10^7	pH 7.4; 25 ºC	(28)
Human Prx5	Prx5	7.0×10^7	pH 7.8; 25 ºC	(29)
M. tuberculosis AhpE	AhpE	1.9×10^7	pH 7.4; 25 ºC	(30)

Table adapted from (31)

H. pullorum prx2 seems to be an essential gene for the survival of the bacterium under the tested *in vitro* conditions. Interestingly, in *H. pylori* the *ahpC* is a key gene, as the *H. pylori ahpC* mutants were only obtained under extremely low oxygen conditions, showed impaired ability to growth under normal microaerobic conditions, and presented the highest impairment for mice colonization relatively to

the correspondent gene mutations in other bacteria. *H. pullorum prx2* was shown to be up-regulated in response to peroxynitrite, and like its AhpC homolog in *S. typhimurium*, *M. tuberculosis*, and *H. pylori*, has peroxynitrite activity. The activity of Prx2 towards peroxynitrite is, however, slightly lower than that reported for other bacterial homologs (Table 7.1).

The *H. pullorum prx3* mutant strain was not possible to be isolated, in contrast to what was previously observed for the *prx3* homologs in *H. pylori* and in other bacteria. Among the three *H. pullorum* peroxiredoxins, Prx3 exhibited the highest peroxynitrite activity, which indicates an important role of Prx3 in peroxynitrite detoxification. Also, *H. pullorum* Prx3 showed a similar value of peroxynitrite activity than the one previously observed for its *M. tuberculosis* Tpx homolog (Table 7.1). *H. pullorum* Prx3 is up-regulated by hydrogen peroxide stress, which indicates a role of this protein in protection against hydrogen peroxide stress. In accordance, *E. coli* and *H. pylori* Tpx were previously reported to reduce hydrogen peroxide and to confer protection against hydrogen peroxide (see Chapter 3, section 3.5).

Figure 7.1 summarizes the studied systems that protect *H. pullorum* against host antimicrobials.

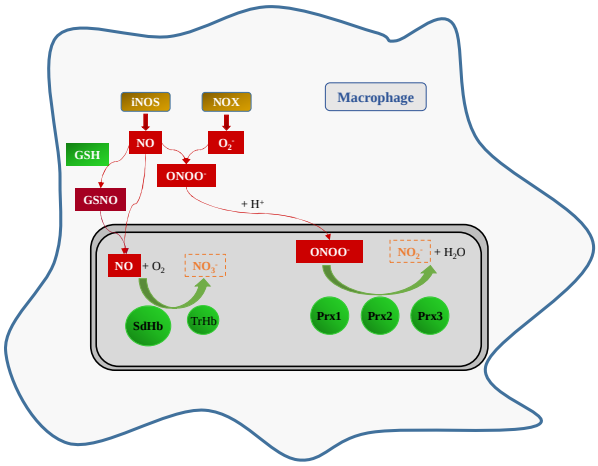

Figure 7.1 Schematic representation of the *H. pullorum* defences against nitrosative stress. The single domain haemoglobin (SdHb) protects the phagocytised *H.*

pullorum cells from nitrosative injury through detoxification of the nitric oxide (NO) produced by iNOS or released from other reactive nitrogen species, such as nitrosoglutathione (GSNO). The *H. pullorum* truncated haemoglobin (TrHb) seems also to be involved in NO protection, by a mechanism similar to that of SdHb. *H. pullorum* uses three peroxiredoxins, (Prx1, Prx2 and Prx3, to convert peroxynitrite ($ONOO^-$) to form nitrite (NO_2^-) and water (H_2O). The scavenging enzymes are represented in green and the damaging compounds are represented in red. Superoxide: O_2^-; phagocyte NADPH oxidase: NOX.

Concerning *H. pylori*, this bacterium was reported to have two nitroreductases, RdxA and FrxA that until now have been described as involved in metronidazole activation. Also, mutations in these enzymes have been associated with *H. pylori* resistance to metronidazole (32). In this work, a new function for one of the *H. pylori* nitroreductases as a defence mechanism against nitrosative stress nitrosative stress was revealed. The biochemical and kinetic characterization of the protein FrxA proved that, apparently, is a typical nitroreductase, binding FMN and reducing a large range of nitro-substituted compounds. The fact that the enzyme is active for nitrocompounds under aerobic conditions, showed that FrxA belongs to the family of the oxygen-insensitive or type I nitroreductases (see Chapter 3, section 3.3). *H. pylori* FrxA reduces nitrofurantoin and nitrofurazone, with activity values that are within the range of activities usually observed for bacterial nitroreductases (Table 7.2). Sisson and co-workers showed that overexpression of *H. pylori frxA* conferred nitrofurantoin and nitrofurazone reductase activity to a nitroreductase-deleted *E. coli* strain, and that inactivation of *frxA* increased the resistance of *H. pylori* to nitrofurantoin. The activity toward nitrofurans herein determined is similar to the ones previously reported for *H pylori* FrxA.

Table 7.2 Activity for nitrofurantoin and nitrofurazone reduction by bacterial nitroreductases

		Activity (μmol·min^{-1}·mg^{-1} of protein)			
Organism	**Enzyme**	**Nitrofurantoin**	**Nitrofurazone**	**Metronidazole**	**References**
Bacillus subtillis	NfrA1	ND	97	ND	(33)
Escherichia coli	NfsA	82	73	ND	(34)
	NfsB	21*	13*	ND	(35)
Helicobacter pylori	RdxA	0.0010	0.0019	9.0	(36, 37)
	FrxA	2.0	0.5	< 0.0002	(37)
		0.5	1.1	0.6	This study
Staphylococcus aureus	NfrA	15	20	ND	(38)
	NtrA	14	15	ND	(39)

* NADH was used as electron donor instead of NADPH. ND - not determined. Table adapted from (40).

The expression of FrxA in cells of *E. coli* promoted the activation of metronidazole; however, the isolated FrxA exhibited no metronidazole reductase activity (37). We showed that FrxA reduces metronidazole, indicating the *in vivo* involvement of *H. pylori* FrxA in metronidazole activation, which is consistent with the presence of mutations in *frxA* of metronidazole resistant *H. pylori* strains. Nevertheless, the metronidazole activity of FrxA is approximately 10-fold lower comparatively to RdxA (Table 7.2), which corroborates the much higher frequency of resistance to metronidazole in *H. pylori* strains containing *rdxA* mutations.

In bacteria, the toxic compound nitrosoglutathione (GSNO), that results from the reaction between the scavenging tripeptide glutathione and NO, promotes nitrosylation of the cysteine residues of proteins thus compromising protein function and bacterial survival (see Chapter I, section 1.3). Enzymes such as the *E. coli* glutathione-dependent formaldehyde dehydrogenase (FALDH) and the nitroreductase NtrA of *S. aureus* were previously shown to reduce GSNO and protect the strain from GSNO toxicity (39).

Besides the typical capacity of nitroreductases to reduce nitrocompounds, *H pylori* FrxA was also shown able to reduce the toxic S-nitrosothiol compounds GSNO, S-nitrosocysteine and 3-nitrotyrosine, the later a product of tyrosine nitration that usually occurs in the tyrosine residues of proteins exposed to nitrosative stress.

From all the substrates tested in our study, FrxA showed the lowest affinity for 3-nitrotyrosine and the highest affinity for the S-nitrosothiols (Chapter IV, Table 4.2). When compared with other microbial GSNO reductases, such as the *S. aureus* NtrA (39) and *E. coli* FALDH (41), FrxA has the highest affinity for GSNO, and in contrast with *S. aureus* NtrA, FrxA exhibits a higher efficiency towards GSNO than for nitrocompounds. All these observations lead us to conclude that the reduction of S-nitrosothiols may be the main function of FrxA. Moreover, as *S. aureus* NtrA and *E. coli* FALDH were not shown to metabolize S-nitrosocysteine, the capacity to reduce both the S-nitrosothiol compounds S-nitrosocysteine and GSNO seems to be a particular feature of *H pylori* FrxA.

The transcription of *frxA* was induced in response to nitrosative stress generated by GSNO and spermine-NONOate. FrxA was shown to afford protection against nitrosative stress, as the deletion of *frxA* lead to a dramatic decrease in the GSNO reductase activity of *H. pylori* cells, and conferred *in vitro* growth resistance to GSNO. Similar observations were reported for *S. cerevisiae* (41), *Haemophilus influenzae* (42) and *S. pneumoniae* (43) FALDH mutants. Also, the *S. aureus* NtrA mutant strain was shown to be more sensitive to GSNO (39). The herein reported ability of FrxA to protect against nitrosative stress and to confer virulence upon mice infection is possibly related to the enzyme capacity to detoxify S-nitrosothiols. As some proteins that are essential for *H. pylori in vivo* survival and pathogenesis, such as urease and AhpC, were reported to be S-nitrosylated and inactivated upon exposure to nitrosative stress (12), the enzyme FrxA may constitute an important detoxifying mechanism in this bacterium. Similarly to FrxA, the NorH (44) and AhpC (45) enzymes, that detoxify NO and peroxynitrite, respectively, contribute to the *H. pylori* successful colonization of mice stomach. Moreover, NorH afforded bacterial protection against iNOS-mediated macrophage killing. Therefore, NorH and FrxA constitute important enzymes for the *H. pylori* resistance to nitrosative stress and virulence.

Altogether this work unveiled some of the enzymes that permit *Helicobacte*r species to sustain and overcome the antimicrobials produced by the host immunity.

7.3 References

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